Regulation of apoptosis in uterine epithelial cells and ovarian cancer cells by the cGMP/ protein kinase G signaling pathway

Chan Siu Lan

Supervisor: Prof. Ronald R. Fiscus

A thesis submitted in partial fulfillment of the requirements

for the degree of Master of Philosophy in Physiology

Department of Physiology

July 2003


香港中文大學
圖書館系統
0 3 JUN 2004
UNIVERSITY
LIBRARY SYSTEM
THE CHINESE UNIVERSITY OF HONG KONG

Abstract

Apoptosis is now becoming recognized as an important event in the development and function of the reproductive system. The present study is aimed at determining the role of the nitric oxide (NO), soluble guanylyl cyclase (sGC)/cGMP/protein kinase G (PKG) signaling pathway in the regulation of apoptosis in two types of female reproductive cells, uterine epithelial cells and ovarian cancer cells. The effects on apoptosis of a NO donor, sGC activators and inhibitors, and PKG activators and inhibitors were determined in cultures of a rabbit immortalized uterine epithelial cell line, HRE-H9 cell, and a human ovarian epithelial cancer cell line, A2780s cells. The effects of the NO donor were also determined in primary cultures of mouse uterine epithelial cells. A new quantitative ultrasensitive technique using capillary electrophoresis with laser-induced fluorescent detector (CE-LIF) was used in some of the experiments as a novel way to accurately measure the levels of apoptotic DNA fragmentation (an index of apoptosis).

The NO donor sodium nitroprusside (SNP) concentration-dependently induced apoptosis in HRE-H9 cells and primary mouse uterine epithelial cells, indicating that these cells are susceptible to the toxic/pro-apoptotic effects of SNP at higher concentrations (0.5-1.0 mM). Two sGC inhibitors, NS2028 and ODQ were used to inhibit basal sGC activity and both were found to increase apoptotic DNA

fragmentation in HRE-H9 cells. The data suggest that the basal activity of sGC and basal cGMP levels may be important for preventing spontaneous apoptosis. A cell permeable cGMP analogue, 8-bromo-cGMP, which directly activates PKG, prevented the ODQ-induced apoptosis in HRE-H9 cells, suggesting that a lowering of basal PKG activity resulting from the sGC inhibition may have been responsible for the induction of apoptosis. Direct inhibition of PKG activity with KT5823 increased apoptotic DNA fragmentation in HRE-H9 cells. These data suggested that basal cGMP/PKG may be necessary to prevent spontaneous development of apoptosis in these uterine epithelial cells.

Increasing the activity of sGC above basal levels has been shown to exert either anti-apoptotic or pro-apoptotic effects, depending on the type of cells and the experimental conditions. The present study determined if activators of sGC and PKG affect serum-deprivation-induced apoptosis in HRE-H9 cells, using a direct, non-NO activator of sGC, YC-1, and two cell-permeable direct activators of PKG, 8-pCPT-cGMP and Sp-8-Br-PET-cGMPS. YC-1, 8-pCPT-cGMP and Sp-8-Br-PET-cGMPS all enhanced the apoptotic DNA fragmentation induced by serum deprivation, measured by CE-LIF.

The present studies thus demonstrated that under basal unstimulated conditions, basal activities of sGC and PKG were shown to be protective to the

uterine epithelial cells. However, when the sGC and PKG activities were stimulated, elevating cGMP levels and PKG activity above the basal levels, the effects of the sGC/cGMP/PKG pathway seems to switch from anti-apoptotic to pro-apoptotic.

XIAP, one of the proteins in the inhibitor of apoptosis (IAPs) family, has been demonstrated to suppress apoptosis in human ovarian epithelial cancer cells. The present study hypothesized that the basal sGC activity in human ovarian epithelial cancer cells exerts anti-apoptotic effects. These effects may involve the regulation of XIAP expression. The sGC inhibitor ODQ was used to determine the effects of basal sGC activity on the levels of apoptosis and XIAP expression in a cisplatin-sensitive ovarian cancer cells line, A2780s cells. Exposure of A2780s cell to ODQ caused a concentration-dependent increase in apoptosis as measured by Hoechst nuclear staining. The increase in apoptosis was accompanied by a significant decrease in XIAP protein content. Overexpression of XIAP caused a concentration-dependent protection of the A2780s cells against ODQ-induced apoptosis. The data suggested that cGMP at basal levels may be involved in preventing spontaneous entry into a pro-apoptotic pathway in the ovarian cancer cells and that down-regulation of XIAP may have been an important element in the ODQ-induced apoptosis.

摘要

細胞凋亡現在被逐漸認爲是生殖系統髮育和發揮作用時的一個重要事件。本研究着重于闡明一氧化氮(NO) ， 水溶性烏苷酸環化酶抑制劑(sGC)和蛋白激酶 G (PKG)信號途徑在調節雌性生殖細胞凋亡。將利用兔子宮上皮細胞(HRE-H9)和人卵巢上皮癌細胞(A2780s)來說明 NO 供體，sGC 激活劑，抑制劑，和 PKG 激活劑，抑制劑，對凋亡的作用中的角色。

研究發現，NO 供體，硝基氫氰酸(SNP)在子宮上皮細胞(HRE-H9)和原代小鼠子宮上細胞上，劑量依賴性地引起凋亡。本室以前研究表明環烏苷單磷酸/蛋白激酶 G (cGMP/PKG) 的激活，可抑制受壓的 PC12 細胞的凋亡。因此，我們進一步研究 cGMP/PKG 在 HRE-H9 細胞凋亡調節中的功用。激光誘發螢光檢測毛細管電泳(CE-LIF)一項新的定量超靈敏技術的測量表明兩種水溶性烏苷酸環化酶(sGC)抑制劑，NS2028，ODQ，都可引起 HRE-H9 細胞的凋亡 DNA 片段。一種可進入細胞，並可激活蛋白激酶 G 的 cGMP 類似物，8-bromo-cGMP(8-溴一環烏苷單磷酸)，可阻斷 ODQ 引發的 HRE-H9 細胞凋亡。我們將進一步研究 PKG 對這些細胞凋亡的調節。PKG 抑制劑，KT5823，可升高 HRE-H9 細胞凋亡 DNA 片段。這些數據表明，基態水平的 cGMP/PKG 對阻止子宮上皮細胞自源性凋亡發展是必需的。

但是 sGC 激活在某些細胞中表現出促凋亡影響。本研究將使用一種直接的，非 NO 的 sGC 激活制 YC-1，和三種直接的，細胞透性的 PKG 激活劑，8-Br-cGMP，

8-pCPT-cGMP 和 Sp-8-Br-PET-cGMPS，來研究是否 sGC 或 PKG 激活劑可影響去血清引發的 HRE-H9 細胞的凋亡。CE-LIF 檢測結果表明 8-pCPT-cGMP 和 Sp-8-Br-PET-Cgmps 都可增強去血清引起的凋亡 DNA 片段。

本研究証明在基態的非刺激條件下，基態活性的 sGC，cGMP 和 PKG 對子宮上皮有保護作用。但是，當 sGC，cGMP 和 PKG 活性被激活，並提高基態的 cGMP 水平和 PKG 活性，sGC/cGMP/PKG 通路似乎可將抗凋亡狀態轉變成促凋亡狀態。

XIAP，一種屬於凋亡抑制劑家族(IAP)的蛋白成員，被証明可抑制人卵巢癌上皮細胞凋亡。至今爲此，尚未有報導關於 sGC 對凋亡調節的影響，以及 XIAP 在卵巢上皮癌細胞中表達。我們猜想基態 sGC 活性可在人卵巢上皮癌細胞中表現出抗凋亡的影響，sGC/cGMP 信號通路的抗凋亡影響可能參與調節細胞內 XIAP 的表達。我們在本研究中確認了 sGC 抑制劑 ODQ 對順鉑敏感化的卵巢癌細胞(A2780s)的凋亡和 XIAP 表達的影響。Hoechst 染色結果表明 ODQ 劑量依賴性地引起 A2780s 細胞凋亡。與此同時 ODQ 處理伴隨着顯著性的 XIAP 降低和輕微的β-tubulin(β-管蛋白) 降低，而對 GAPDH 是稳定和無顯著性影響。過量表達 XIAP 可劑量依賴性地保護 A2780s 細胞對抗 ODQ 引起的凋亡。這表明，基態水平的 cGMP 可能參與阻止卵巢癌細胞自發進入促凋亡通路。這些數據指出調低 XIAP 可能是 ODQ 一引起的 A2780s 細胞凋亡之中的一重要事件。

總的來說，細胞的 sGC 和 PKG 活性及 cGMP 水平在雌性生殖細胞中是嚴格

調控的，其對調節凋亡可能起重要作用。提高 sGC，PKG 和 cGMP,基態水平可能會從抗凋亡影響轉變成促一凋亡影響。ODQ，一種 sGC 抑制劑，可引起子宮上皮細胞和卵巢癌細胞凋亡。

Acknowledgement

I would like to express my gratefulness to those people who have helped and supported me through the whole course of the study in the Chinese University of Hong Kong.

I would like to express my deepest gratitude to my supervisor, Prof. Ronald R. Fiscus, for his excellent guidance, invaluable advice, strong support, great patience and continuous encouragement throughout the process of my study. As a scientist who worked in NO/cGMP/PKG pathway for about twenty years, he gave me a lot of background knowledge, which is extremely beneficial to my work during my post-graduate program and also to my future work. His enthusiastic and precise manner toward science gave me great impression, which will always inspire me in my future research. I also want to sincerely thank our technician Jessie Y.C. Yuen for her technical support and discussion of the findings.

I would also like to thank Prof. Benjamin K. Tsang, our collaborator, for his guidance on my research on human ovarian epithelial cells in Ottawa, Canada. I can never forget his enthusiasm. I feel so lucky that I can go to Prof. Tsang's laboratory in Ottawa and stay there for five months to conduct my research experiment. I would like to give my thanks to the people who have worked in Prof. Tsang's laboratory,

Andree Gruslin, Jin Yi Jiang, Qing Qiu, Xiaojuan Yan, Mohammad Reza Abedini, Carmen Cheung, Michael Fraser, Arezu Jahani-Asl and Ming Yan Yang. Their friendship and help gave me confidence in conducting research in a new area and environment. It was really a great pleasure to work with them.

I am sincerely grateful to Mr. Anthony E. James and John Tse in the Laboratory Animal Services Center, The Chinese University of Hong Kong for the arranging and supply of animal to us. A lot of help was also given to me from many persons from the Department of Physiology. Many technical support and research instruments came from the Multidiscipline Laboratory of the Faculty of Medicine.

Financial Support had been provided as studentship from the graduate school. I would like to give special thanks to Graduate School Bursary Sub-Committee of The Chinese University of Hong Kong, for approving my application for the grant for Overseas Academic Activities and grant for attending Conference (1st Batch of Awards, 2002-2003).

Last but not the least, I would like to express my deepest gratitude to my parents. Without their unconditional support, endless encouragement and love, I could never overcome all the difficulties in my life.

Publications

Journal articles

1. Fiscus, R.R., Yuen, J.P., Chan S.L., Kwong, J.H. and Chew, S.B. Nitric oxide and cyclic GMP as pro- and anti-apoptotic agents. **J. Card. Surg.** 2002; 17(4), 336-339.

2. Chan, S.L. and Fiscus, R.R. Apoptotic DNA fragmentation caused by sodium nitroprusside, a nitric oxide donor, in uterine epithelial cells: Ultrasensitive quantitation using the new capillary electrophoresis/laser-induced fluorescence (CE-LIF) technology. (Submitted to Toxicology and Applied Pharmacology)

3. Chan, S.L. and Fiscus, R.R. Guanylyl-cyclase inhibitors NS2028 and ODQ and protein-kinase-G inhibitor KT5823 trigger apoptotic DNA fragmentation in an immortalized uterine epithelial cell line: anti-apoptotic effects of basal cGMP/PKG. (Submitted to Molecular Human Reproduction)

4. Chan, S.L. and Fiscus, R.R. Direct, prolonged activation of soluble guanylyl cyclase by YC-1 or protein kinase G by cGMP analogs enhances the levels of apoptosis in an immortalized uterine epithelial cell line, HRE-H9 cells. (Submitted to Biology of Reproduction)

5. Chan, S.L., Fraser, M., Fiscus, R.R. and Tsang, B.K. Down-regulation of XIAP protein content and induction of apoptosis by the soluble guanylyl cyclase inhibitor ODQ in human ovarian cancer cells (In preparation)

Conference Presentations

1. Chan, S.L., Fiscus, R.R. (2001). Inhibition of sol. guanylyl cyclase or protein kinase G causes apoptotic DNA fragmentation in unstressed HRE-H9 cells: Quantitation by new ultrasensitive CE-LIF technology. The Twenty-First Annual Conference of the Hong Kong Society of Neuroscience. December 7-8, 2001. The Chinese University of Hong Kong. (Poster)

2. Chan, S.L., Fiscus, R.R. (2002). ODQ, guanylyl cyclase inhibitor, and KT5823, protein kinase G (PKG) inhibitor, trigger onset of apoptotic DNA fragmentation in uterine epithelial cells: Role of basal cGMP as anti-apoptotic mechanism. XIVth World Congress of Pharmacology. July 7-12, 2002. San Francisco, California. (Poster)

Table of contents

Chapter 3: Literature Review

List of Figures

List of Table and Diagram

Abbreviations

AIDS	Acquired Immunity Deficiency Syndrome
AIF	Apoptosis Inducing Factor
ALS	Amyotrophic Lateral Sclerosis
ANP	Atrial Natriuretic Peptide
Apaf-1	Apoptotic Protease Activating Factor
BIRs	Baculovirus IAP Repeats
BNP	Brain Natriuretic Peptide
CaM-PDE, PDE1	Ca^{2+}-calmodulin-dependent phosphodiesterase
CE	capillary electrophoresis
CGB-PDE, PDE5	cGMP-specific cGMP-binding phosphodiesterase
cGI-PDE, PDE3	cGMP-inhibited Phosphodiesterase
cGMP	Cyclic Guanosine Monophosphate
cGS-PDE, PDE2	cGMP-stimulated Phosphodiesterase
CHO	Chinese Hamster Ovary
CNG	Cyclic Nucleotide-Gated Cation Channel
Cyt c	Cytochrome c
DED	Death Effector Domain
DMEM	Dulbecco's Modified Eagle Medium
ER	Endoplasmic Reticulum
FADD	Fas associated death domain
FBS	Fetal Bovine Serum
GTP	Guanosine Triphosphate
HBSS	Hank's Balanced Salt Solution
HIAP-1	Human Inhibitor of Apoptosis Protein-1
HIAP-2	Human Inhibitor of Apoptosis Protein-2
HRP	Horseradish Peroxidase
IAP	Inhibitor of Apoptosis Protein
IL-1β	Interleukin-1β
iNOS	Inducible Nitric Oxide Synthase
LIF	laser-induced fluorescence
NAIP	Neuronal Apoptosis Inhibitory Protein
NEAA	Nonessential Amino Acid
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NS2028	4H-8bromo-1, 2,4-oxadiazola (3,4-d) benz (b-1,4) oxazin-1-one

ODQ	1H-[1,2,4] oxadiazolo [4,3, -a] quinoxalin-1-one
$ONOO^-$	Peroxynitrite
P/S	Penicillin-Streptomycin
PDE	Phosphodiesterase
pGC	Particulate Guanylyl Cyclase
PKG	Protein Kinase G/cGMP-dependent Protein Kinase
PKG-1	Protein Kinase G Type 1
sGC	Soluble Guanylyl Cyclase
SNP	Sodium Nitroprusside
TNF	Tumour Necrosis Factor
TUNEL	Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End-Labeling
XIAP	X-linked Inhibitor of Apoptosis Protein
YC-1	3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole

Chapter 1: Introduction

1.1 Major objective and long-term significance

The major objective of this research project was to investigate the functional roles of the nitric oxide (NO)/cGMP/protein kinase G (PKG) signaling pathway in the regulation of apoptosis and XIAP protein content in cells of the female reproductive system. It is hoped that the findings of this study will provide a better understanding of the fundamental mechanisms of signal tranduction that regulate apoptosis. It is also hoped that the improved understanding of apoptosis can lead to the development of new and better drugs for treating diseases and condition caused by dysregulation of apoptosis, including cancer, dysmenorrhea, endometriosis and infertility.

1.2 Biological significance of apoptosis

Apoptosis, also called programmed cell death, has gained widespread attention in recent years due to its newly discovered role in a variety of physiological and pathological processes (Thompson, 1995; Jacobson et al., 1997). Apoptosis is an active physiological mode of cell death, in which the cells execute a program of their own demise and subsequent disposal. This apoptotic pathway is apparently shared by all multi-cellular organisms and is critical for the successful development of multiple tissues during

embryonic development and the maintenance of normal tissue homeostasis after development.

Cells have the ability to self-destruct by activation of an intrinsic cellular suicide program when they are no longer needed or become damaged irreparably after exposure to stressful stimuli or infectious agents, or when they no longer receive signals from specific survival factors (Kerr et al., 1972). Therefore, apoptosis is important to many physiologic processes such as cell selection in development and immunologic responses (Pospisil et al., 1995), in the control of organ size in maturation and regeneration (Raff, 1996) and in normal cell turnover throughout the organism (Thompson, 1995). The regulation of apoptosis is thus critical for many processes, including development, the defense against pathogens and the prevention of proliferation of transformed cells.

Dysregulation of apoptosis may underlie the etiology of several diseases. It is known that apoptosis plays an important role in the maintenance of physiological homeostasis in response to stimuli that indicate that a cell is potentially harmful or abnormal (Steller, 1995; Thompson, 1995). When the apoptosis machinery fails, abnormal cells can survive, and unopposed tissue growth, as in the case of cancer, can result. Failure of dividing cells to initiate apoptosis in response to DNA damage has been implicated in the development and progression of cancer and leukemia. Thus, carcinomas may be caused or promoted in part by factors inhibiting cell death. On the other hand, increased levels of

apoptosis may be involved in other diseaseses, such as neurodegenerative diseases (Kure et al., 1991).

1.3 Importance of apoptosis in the study of female reproduction system

Apoptosis is now recognized as an important event in the development and function of the reproductive system (Gosden and Spear, 1997). Apoptosis is important for the normal function of both uterine epithelial cell and ovarian epithelial cell in the female reproductive system. (For more detail, see literature review)

1.4 Specific aims of the present project

The specific aims of this project were focused on resolving the following questions:

(1) Is NO a pro-apoptotic agent in uterine epithelial cells? Can the levels of apoptotic DNA fragmentation induced by NO be accurately quantified?

Data from two recent studies have suggested that NO may have toxic effects in uterine epithelial cells, leading to increased apoptosis. First, isolated slices of human endometrial tissue show increased apoptosis (assessed by TUNEL staining) when incubated *in vitro* with L-arginine, the precursor of NO biosynthesis (Castro et al., 2002). Second, a human endometrial carcinoma cell line (RL95-2 cells), when exposed to sodium

nitroprusside (SNP), a NO donor, showed decreased cell survival, assessed by the MTT technique, and increased caspase-3 activity, indicator of apoptosis (Li et al., 2001b). These finding suggested that NO is pro-apoptotic to uterine epitheial cells. However, to our knowledge, there have been no previous reports providing an accurate measure of apoptotic DNA fragmentation to quantify the pro-apoptotic effects of NO in uterine epithelial cells. The purpose of the present study was to accurately quantify the effects of the NO donor SNP on apoptotic DNA fragmentation in uterine epithelial cells, using both a rabbit immortalized epithelial cell line (HRE-H9 cells) and primary cultures of mouse uterine epithelial cells and two techniques for assessing DNA fragmentation, agarose gel electrophoresis and the new quantitative capillary electrophoresis with laser-induced fluorescence (CE-LIF).

(2) Are basal levels of cGMP and PKG activity exerting anti-apoptotic effects in uterine epithelial cells?

NO exerts anti-apoptotic effects in certain cells, either through mechanisms involving activation of soluble guanylate cyclase (sGC) and subsequent elevation of cGMP levels or, in some cells, through mechanisms independent of sGC (Kim et al., 1997; Kolb, 2000; Fiscus, 2002; Fiscus et al., 2002). Endogenous NO synthesis or exposure to low levels of NO donors has now been shown to inhibit apoptosis in a number of different kinds of cells, including B lymphocytes (Mannick et al., 1994; Genaro et al., 1995),

eosinophils (Beauvais et al., 1995), ovarian follicles (Chun et al., 1995), endothelial cells (Dimmeler et al., 1997) and rat cerebellar granule cells and cortical cells (Pantazis et al., 1998; Fernandez-Tome et al., 1999). In some of these cells, the anti-apoptotic actions of NO were shown to be dependent on the cGMP elevations (Beauvais et al., 1995; Chun et al., 1995; Genaro et al., 1995), while in other cells, the anti-apoptotic mechanism of NO was independent of cGMP (Mannick et al., 1994).

Previously data from the laboratory of Professor Fiscus had shown that two natriuretic peptides, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), which activate particulate guanylyl cyclase (pGC) and cause prolonged elevations of cGMP levels, are potent and effective inhibitors of apoptotic cell death caused by serum-deprivation-induced stress in PC12 cells, resulting in a significant prolongation of cell survival (Fiscus et al., 2001b). Furthermore, 8-Br-cGMP, a cell-permeable analog of cGMP that directly activates PKG, was also found to inhibit apoptosis in stressed PC12 cells. Thus, cGMP and PKG appeared to be intimately involved in the prevention of apoptosis and the prolongation of cell survival, at least in certain types of cells, like PC12 cells. Many other neural cells have also been found to possess a similar anti-apoptotic/pro-survival pathway involving the cGMP/PKG signaling pathway (for Reviews, see Fiscus, 2002; Fiscus et al., 2002). Furthermore, in some neural cells, such as N1E-115 and NG108-15 cells, even the basal levels of cGMP and basal activity of PKG appear to be sufficient to cause anti-apoptotic effects, protecting these cells against a spontaneous

development of apoptosis (Yuen and Fiscus, 2001; Fiscus, 2002). The present study was designed to determine if basal cGMP levels and basal PKG activity are involved in the control of apoptosis in uterine epithelial cells, using an immortalized cell line, the HRE-H9 cells.

(3) What are the effects on apoptosis of elevating cGMP levels and PKG activity in uterine epithelial cells?

The effects of sGC on the survival of mammalian cells appear to be different in different cell types and different experimental conditions. For example, activation of sGC has been shown to exert pro-apoptotic effects in some types of cells, including cardiomyocytes (Taimor et al., 2000), colon tumor cells (Thompson et al., 2000) and vascular smooth muscle cells (Pollman et al., 1996). However, there are also reports showing protective effects of sGC on the survival of other types of cells, such as neural cells (Garthwaithe and Garthwaithe, 1988; Garthwaite et al., 1995; Estevez et al., 1998; Thippeswamy and Morris, 1997; Fiscus, 2002; Fiscus et al., 2002), leukemia cells (Flamigni et al., 2001) and liver cells (Li et al., 2000). In the present study, we determined the effects of a direct, NO-independent sGC activator, YC-1, on the apoptosis induced by serum deprivation in HRE-H9 cells. NO was not used as an activator of sGC in the present study because it is known to activate a number of toxic/pro-apoptotic pathways that are independent of the sGC/cGMP pathway (Fiscus, 2002; Fiscus et al., 2002). The effects of

three cGMP analogues, 8-Br-cGMP, 8-pCPT-cGMP, Sp-8-Bromo-PET-cGMPS, all of which are cell-permeable, direct activators of PKG, were also determined on the induction of apoptosis in HRE-H9 cells.

(4) What are the effects of basal sGC activity on the regulation of apoptosis and the expression of XIAP in ovarian cancer cells?

Basal activities of sGC and a downstream target protein in the cGMP signaling pathway, protein kinase G (PKG), appear to be important for the survival of uterine epithelial cells (Chan and Fiscus, 2002) and several neural cell lines (Yuen and Fiscus, 2001; Fiscus, 2002). There may be a similar involvement of basal sGC and PKG activities in regulating the survival of ovarian cancer cells. In ovarian cancer cells, the anti-apoptosis protein XIAP is known to play an important role in preventing apoptosis, thus prolonging the survival. For example, infection of cisplatin-sensitive ovarian cancer cells with adenoviral sense XIAP complementary DNA resulted in overexpression of XIAP and markedly attenuated the ability of cisplatin to induce apoptosis (Li et al., 2001a). A working hypothesis of the present study was that at least part of the anti-apoptotic effects of the sGC/cGMP signaling pathway may involve the regulation of XIAP expression. The present study determined the effects of the sGC inhibitor ODQ on the levels of apoptosis and XIAP expression in a cisplatin-sensitive ovarian cancer cell line, the A20780s cells.

1.5 Experimental Approaches

(1) Capillary Electrophoresis with Laser-Induced Fluorescence Detector (CE-LIF)

The present study utilized CE-LIF to determine the levels of apoptosis in both immortalized rabbit uterine epithelial cells and primary cultures of mouse uterine epithelial cells. Internucleosomal fragmentation of DNA is a hallmark of apoptosis in many cells (Compton, 1992). Unfortunately, the conventional technique for measuring apoptotic DNA fragmentation, i.e. DNA laddering by agarose gel electrophoresis, is a relatively insensitive method that typically requires 1-10 million cells per sample for the detection of apoptosis (Fiscus, 2002; Fiscus et al., 2002). Furthermore, DNA laddering on agarose gels is not quantitative, making it difficult to objectively analyze the effects of pro- and anti-apoptotic agents.

Recently, our laboratory has developed an ultrasensitive quantitative technique for quantifying apoptotic DNA fragmenation in neural cells (Fiscus et al., 2001b; Fiscus, 2002; Fiscus et al., 2002), uterine epithelial cells (Fiscus et al., 2001a) and epididymal epithelial cells (Cheuk et al., 2002) using capillary electrophoresis (CE) coupled with the new, ultrasensitive laser-induced fluorescence detector (LIF). This new CE-LIF technology provides a more than 1,000-fold improvement in the sensitivity of measuring DNA fragmentation, as compared to the conventional agarose gel technique. Furthermore, both

internal and external DNA standards as well as computer analysis of CE-LIF electropherograms are used to accurately quantify the levels of apoptotic DNA fragmentation caused by proapoptotic agents as well as the percentage of inhibition of apoptosis caused by anti-apoptotic agents.

This technology allows, for the first time, accurate measurement of apoptotic DNA fragmentation in experiments using limited, small numbers of cells, such as primary cultures or extremely small punch biopsies. Besides, CE-LIF has the advantage that the resulting data can be analyzed by objective methods using modern statistical techniques. Furthermore, because of the exquisite sensitivity of the CE-LIF technology, which requires fewer cells for analysis of apoptotic DNA fragmentation, larger numbers of treatment conditions and replications of these conditions can be tested.

(2) Nuclear Staining (Hoechst Staining)

The morphology of apoptosis is strikingly similar in different cell types. As recognized by electron microscopy, the nuclear chromatin aggregates into uniformly dense masses, which abut on the nuclear membrane as the cytoplasm begins to compact. These changes are followed by breaking up of the nucleus into discrete fragments together with fragmentation of the cell (Wyllie et al., 1980). In our study, apoptosis of human ovarian epithelial cancer cells, A2780s cells, was quantitatively measured by nuclear staining.

Apoptotic nuclei with condensed chromatin was characterized and quantified by staining with the DNA-specific dye Hoechst 33248. Nuclear staining was observed and photographed using a Zeiss fluorescence microscope (magnification 400X). Cells with typical apoptotic nuclear morphology (nuclear shrinkage, condensation, and fragmentation) were identified and counted, using randomly selected fields on numbered photographic slides to avoid bias. A minimum of 600 cells per treatment was counted in each treatment.

Chapter 2 Materials and Methods

2.1 General experimental methods

2.1.1 Culture of cells

2.1.1.1 Culture of rabbit immortalized uterine epithelial cells

The HRE-H9 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with phenol red containing 4% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, in the presence of 5% CO_2 at 37°C, which was a slight modification of the method of Wing and coworkers (Wing et al., 1998). During passaging, the cells were washed with Hank's Balanced Salt Solution (HBSS, without phenol red) and harvested by trypsinzation using trypsin. The cells were counted using a haemocytometer.

In all experiments, the cells were used between passage 20 and 45. For preparation of experiments using only CE-LIF to determine DNA fragmentation, cells were seeded in medium supplemented with 4% serum in 6-well culture plates at a density of 1 million cells per well. After 24 h, the medium was aspirated and replaced by medium of the different conditions indicated in the results. For the experiments of overcrowding-induced apoptosis, in which both CE-LIF and agarose gels were used to determine DNA fragmentation, cells were plated into 100 mm culture dishes at a density of 5 million cells per dish. Cells were allowed to grow in the presence of 5% CO_2 at 37°C for 24, 36, 48, 60

and 72 h in the same medium as above. For the NO-induced apoptosis in HRE-H9 cells, using both CE-LIF and agarose gels, the cells were also plated into 100 mm culture dishes at a density of 5 million cells per dish and were cultured in medium with 4% serum for 24 h before addition of the NO donor. The cells were incubated with the NO donor sodium nitroprusside (SNP) at 62.5, 125, 250, 500 and 1000 μM in the presence of 5% CO_2 at 37°C for 7 h.

2.1.1.2 Culture of primary mouse uterine epithelial cells

For the primary culture, the endometrial epithelial cells were enzymatically isolated from the C57BL/6J mouse uterus according to the method described by McCormack and Glasser for the isolation of rat uterine epithelial cells (McCormack and Glasser, 1980), except with the modifications introduced by Chan and coworkers for the isolation of ICR mouse uterine epithelial cells (Chan et al., 1997). For each culture preparation, samples of uterus were obtained from 40 C57BL/6J mice (4-6 week). The sliced uteri were incubated in PBS supplemented with 5 mg/ml trypsin and 25 mg/ml pancreatin at 0°C for 60 min and then room temperature for another 45 min. After the enzyme digestion, the conical centrifuge tube containing PBS and the tissue was shaken gently for 30 s. Uterine tissue was carefully removed, and the crude cell suspension was passed through a 70 μm fluorocarbon mesh filter (Spectra/Mesh, Spectrum, Houston, TX). The filtrate was centrifuged at 1000 X g for 5 min. The cell pellet was resuspended in 1 ml PBS and centrifuged again at 1000 X g for 5 min. The washing procedure was then repeated a

second time. After centrifugation, the cell pellet was resuspended in DMEM/Ham's F-12 culture medium containing 10% fetal bovine serum, 1% non-essential amino acid (NEAA), 100 U/ml penicillin, and 100 μg/ml streptomycin. The isolated endometrial cells were plated in 6-well culture plates and incubated at 5% CO_2 at 37°C for 3 days. For the serum-deprivation-induced apoptosis, cells were washed with PBS and incubated in DMEM/Ham's F-12 culture medium without serum for 3 days. For the NO-induced apoptosis, SNP (500 μM, final concentration) was added to the medium (same as above) and incubated for 3 days.

2.1.1.3 Culture of human ovarian epithelial cancer cells

A2780s cells (a cisplatin-sensitive human ovarian epithelial cancer cell line) were maintained at 37°C and 5% CO_2 and 95% air in DMEM/F12 medium, supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, 50 U/ml penicillin, 0.625 g/ml fungizone and 1% non-essential amino acids, as previously reported (Asselin et al 2001). Cells were plated at a density of 5 x 10^4 cells/cm^2 in 6-well plates. After a 24 h cell-plating period, the culture medium was changed and the cells were treated with agonistic monoclonal anti-Fas antibody, ODQ or adenoviral XIAP sense cDNA under serum-free conditions. Serum withdrawal had no significant effect on cell viability during the experimental period, as confirmed by 3-(4,55-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide assay.

2.1.2 Assessment of apoptotic DNA fragmentation

2.1.2.1 DNA extraction

DNA was extracted by the following procedure. Epithelial cells in each plate/well were incubated with lysis buffer (5 mM Tris-HCl, 0.5 % Triton X-100, and 20 mM EDTA, pH 8.0) by adding the lysis buffer directly to the plate/well. The pellets of the floating cells were collected after centrifugation and were added to the corresponding cell lysates. The cell lysates, together with the added floating cells, were mixed and incubated on ice for 15 min. Then, cell lysates were centrifuged at 7000 g for 15 min at 4°C and the supernatants were collected. DNA in supernatant fractions was extracted with phenol (supernatant: phenol, 1:1) and then extracted with phenol: chloroform: isoamyl alcohol (25:24:1). The fragmented DNA was precipitated in absolute ethanol (5:2) and sodium acetate (3 M, pH 5.2) (10:1) at -20°C overnight. The DNA precipitate was collected by centrifugation of the sample at 14,000 rpm for 10 min at 4°C. The DNA pellet was washed in 70% ethanol and centrifuged at 13 000 rpm for 10 min at room temperature. The DNA pellet was then air dried, dissolved in TE buffer (5 mM Tris HCl, pH 8.0, 20 mM EDTA), treated with RNase (10 mg/ml) at room temperature for 1 h to remove RNA.

2.1.2.2 Assessment of apoptotic DNA fragmentation by agarose gels

Apoptotic DNA fragmentation in HRE-H9 cells was analyzed by DNA laddering on both 2% agarose gels and capillary electrophoresis with laser-induced fluorescence

detector (CE-LIF), a technique developed in our laboratory (Fiscus et al., 2001a; Fiscus et al., 2001b; Fiscus 2002, Fiscus et al., 2002) (see below). Because of the limited number of primary uterine epithelial cells that could be collected from the mice, apoptotic DNA fragmentation in primary cultures was analyzed by CE-LIF only. Both floating cells and adherent cells were combined for the assessment of apoptosis. DNA in agarose gels was visualized using ethidium bromide (1 μg/ml) and a UV transilluminator (Sigma).

2.1.2.3 Assessement of apoptotic DNA by CE-LIF

The CE-LIF technique used dsDNA1000 kit (Bio-Rad) to analyze the apoptotic DNA fragments, following a procedure previously developed in our laboratory for quantifying the levels of apoptosis in cultured HRE-H9 uterine epithelial cells (Fiscus et al., 2001a) and neural (N1E-115, NG108-15 and PC12) cells (Fiscus et al., 2001b; Fiscus, 2002; Fiscus et al., 2002). Because the CE-LIF technique is >1,000 times more sensitive than agarose gels, it can measure apoptotic DNA fragmentation in just a few thousand cells, thus making the CE-LIF technique especially useful in experiments that involve primary cultures. Briefly, the procedure used a coated capillary (24 cm X 75 μM), electrophoretic injection at 10 kV for 2 s, run at 2.5 kV for 40 min at 4°C and an argon laser at 488 nm. The kits included SYBR Green I as the dsDNA-specific fluorescent label. Standards of known amounts of a 540 bp DNA fragment were run in the CE-LIF using identical conditions as the samples. The resulting standard curve was then used to calculate the

amount of apoptotic DNA in the 540 bp peaks, based on area under the peak/migration time.

2.1.2.4 Assessement of apoptosis by Nuclear Hoechst 33248 Staining

At the end of the culture period, cells attached to the growth surface were removed by trypsin treatment [trypsin (0.05%), EDTA (0.53mM); 37 °C, 1 min]. Attached and detached cells were pooled, pelleted, and resuspended in 10% neutral-buffered formalin containing Hoechst 33248 dye (12.5 ng/ml). Cells were spotted onto slides for microscopy. Nuclear staining was observed and photographed using a Zeiss fluorescence microscope (magnification 400X). Cells with typical apoptotic nuclear morphology (nuclear shrinkage, condensation, and fragmentation) were identified and counted, using randomly selected fields on numbered photographic slides to avoid bias. A minimum of 600 cells was counted in each treatment group.

2.1.3 Assessement of protein content

2.1.3.1 Protein extraction and Western blot analysis

Cells were pelleted and lysed in ice-cold lysis buffer (pH 7.4) containing 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM NaPPi, 10 % Glycerol and 1% Triton X-100. Protease inhibitors, PMSF (1 mM) and aprotonin (10

μg/μl), as well as 1 mM Na_3VO_4 were added to the lysis buffer freshly. Cell lysates were then sonicated briefly (5 s/cycle, 3 cycles; 0 °C). The sonicates were incubated on ice for 1 h and pelleted by centrifugation (15, 000 x g; 20 min). The supernatant was saved and stored at -20 °C for subsequent analyses. Protein concentration was determined using Bio-Rad DC protein assay kit. Equal amounts of proteins (40 μg) were loaded and resolved by 10% SDS-Polyacrylamide gel electrophoresis (PAGE) and electrotransferred (30V, 16 h) onto nitrocellulose membranes (Bio-Rad). Uniform protein loading was confirmed by comparing the intensity of stained total proteins between lanes of nitrocellulose membranes. The images were then scanned and stored electronically. The intensity of the protein stained in each lane was quantified for future reference or for normalization of Western blot results. Membranes were then blocked (room temperature, 1 h) with 5% Blotto (Tris-HCl (10 mM; pH 8.0), NaCl (150 mM), Tween 20 (0.05%, v/v; TBS-Tween 20) containing skimmed milk (5%; w/v), then incubated overnight with primary antibodies [anti-PKG-Iα/β, (1:1000), anti-XIAP (1:2,000) or anti-GAPDH (1:4000)], and subsequently with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,500 in 5% Blotto; room temperature, 1 h). Peroxidase activity was visualized with an ECL kit (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) after three washes (each 15 min) with TBS-Tween 20 and a single wash (10 min) with TBS [Tris-HCl (10 mM; pH 8.0), NaCl (150 mM)]. Signal intersity was determined densitometrically and normalized against those of total proteins present in the corresponding lane on the membrane using Molecular Analyst software, version 1.5 Bio-Rad (Hercules, CA, USA).

2.1.4 Adenoviral infection of A2780s cells

After 24 h of plating (10^6 cells/60-mm culture dish), cells were infected with an adenoviral full-length XIAP sense cDNA or LacZ at a multiplicity of infection (MOI) of 0, 2.5, 5 or 10 for 48 h, as previously described (Li et al 2001a). Adenovirus infection efficiency at MOI of 5, as determined by an X-gal staining assay against LacZ construct infected cells, was about 90%. The number of apoptotic cells was determined by Hoechst staining. XIAP protein contents were then assessed by Western blot.

2.2 Preparation of solutions

The working solutions and stock solutions were prepared as described below. They are listed alphabetically.

1. 8-Br-cGMP was weighed and and dissolved in deionized water to the concentration required. 8-Br-cGMP was from Calbiochem.
2. Adenoviral full-length Lac Z sense cDNA was diluted in culture medium to the MOI used. Lac Z sense DNA was prepared by the Adenovirus Core Facility, University of Ottawa Neuroscience Research Institute.
3. Adenoviral full-length XIAP sense cDNA was diluted in culture medium to the MOI used. XIAP sense DNA was prepared by the Adenovirus Core Facility, University of Ottawa Neuroscience Research Institute.

4. Agarose gel (2%) was prepared by weighing 0.8 g of agarose to 40 ml of 1X TAE. 1 X TAE was prepared from 50 X TAE. Both agarose and 50 X TAE were from GIBCO BRL.
5. Anti-GAPDH antibody was diluted 1:4000 in Blotto. Anti-GAPDH antibody (ab8245) was from Abcam.
6. Anti-PKG-Iα/β antibody was diluted 1:1000 in Blotto. Anti-PKG-Iα/β antibody was from Calbiochem.
7. Anti-XIAP antibody was diluted 1:2000 in Blotto. Rabbit polyclonal anti-human XIAP antibody was from Trevigen.
8. Blotto (5%) contains Tris-HCl (10 mM; pH 8.0), NaCl (150 mM), Tween 20 (0.05%, v/v; TBS-Tween 20) and skim milk (5%; w/v). Tris-HCl was from Bio-Rad. NaCl was from Sigma, Tween-20 was from Fisher Scientific and skim milk was from Nestle.
9. The DMEM used as the culture medium of the rabbit immortalized uterine epithelial cells, HRE-H9 cells contained phenol red, 4% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. DMEM, fetal bovine serum, penicillin and streptomycin were all from GIBCO BRL.
10. The DMEM/Ham's F-12 used as the culture medium of the primary mouse uterine epithelial cells contained 10% fetal bovine serum, 1% non-essential amino acid (NEAA), 100 U/ml penicillin, and 100 μg/ml streptomycin. DMEM/Ham's F12, fetal bovine serum, NEAA, penicillin and streptomycin were all from GIBCO BRL.

11. The DMEM/Ham's F-12 used as the culture medium of the human ovarian epithelial cancer cells, A2780s cells contained 10% fetal bovine serum, 1% non-essential amino acids (NEAA), 50 U/ml penicillin, 50 μg/ml streptomycin and 0.625 g/ml fungizone. DMEM/Ham's F12, fetal bovine serum, NEAA, penicillin and streptomycin were all from GIBCO BRL. Fungizone was from Life Technologies, Inc./ BRL
12. ECL^{TM} (enhanced chemiluminescence) detected kits was from Amersham Amersham Pharmacia Biotech.
13. Ethanol (70 %) was prepared by diluting absolute ethanal in deionized water. 70% ethanol was used for washing DNA pellet, while absolute ethanol is used with sodium acetate for precipitating DNA. Absolute ethanol was from Merck.
14. Ethidium bromide was weighed and dissolved in deionized water to prepare the stock of 10 mg/ml. 2 μl of stock of ethidium bromide (1 μg/ml) was added to 40 ml of 2% agarose gel for visualization of DNA by using a UV transilluminator. Both ethidium bromide and UV transilluminator were from Sigma.
15. Hank's Balanced Salt Solution (HBSS, without phenol red) was used for washing HRE-H9 cells before passaging or harvesting by trypsinization. HBSS was from Sigma
16. Hoechst 33248 dye was weighed and dissolved in deionized water to prepare the stock of 6.25 μg/μl. 2 μl of Hoechst staining stock solution was diluted in 1 ml of buffered formalin to obtain a final concentration of 12.5 ng/ml. 40 μl of final Hoechst

staining solution in buffered formalin was used for resuspending cell pellets. Hoeschst staining dye was from Sigma

17. Horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:2000 in blotto for anti- PKG-Iα/β and diluted 1:2500 in Blotto for anti-XIAP and anti-GAPDH. Horseradish peroxidase (HRP)-conjugated secondary antibody was from Amersham Amersham Pharmacia Biotech.
18. KT5823 was weighed and diluted in deionized water to the concentration required. KT5823 was from Calbiochem.
19. Lysis buffer (for DNA extraction) contains 5 mM Tris-HCl, 0.5 % Triton X-100 and 20 mM EDTA. The lysis buffer was adjusted to pH 8.0. Tris-HCl and Triton X-100 were from Sigma and EDTA was from GIBCO BRL.
20. Lysis Buffer (for protein extraction) contains 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM NaPPi, 10 % Glycerol and 1% Triton X-100. Protease inhibitors, PMSF (1 mM) and aprotonin (10 μg/μl), as well as 1 mM Na_3VO_4 were added to the lysis buffer freshly. The lysis buffer was adjusted to pH 7.4. All chemicals were from Sigma.
21. Monoclonal agonistic anti-Fas antibody (Fas mAb) was diluted in culture medium to the concentration required. Fas mAb was from Upstate Biotechnology.
22. Mouse IgM was diluted in culture medium to the concentration required. Mouse IgM was from Upstate Biotechnology

23. Neutral-buffered formalin (10%) was used for resuspending and fixing cells for nuclear Hoechst staining. Neutral-buffered formalin was from Sigma.
24. ODQ was weighed and diluted in DMSO first and then diluted to the concentration required in culture medium. ODQ was from Calbiochem. DMSO was from Sigma.
25. Phenol was used for the first step of DNA extraction. DNA was extracted first by adding phenol in a one-to-one ratio to the supernatant of the sample. Phenol was from GIBCO BRL.
26. Phenol: chloroform: isoamyl alcohol (25:24:1) mixture was used for the final extraction of the DNA. The mixture was added in one-to-one ratio to the supernatant of the sample. Chloroform and isoamyl alcohol were from Sigma while phenol was from GIBCO BRL.
27. Phosphate Buffer Saline (PBS) supplemented with 5 mg/ml trypsin and 25 mg/ml pancreatin was used for preparing mouse uterine epithelial cells from mouse uterus by enzymatic digestion. PBS and pancreatin were from GIBCO BRL. Typsin was from Sigma. PBS by itself was used for washing primary mouse uterine epithelial cells before passaging or harvesting by trysinization.
28. RNase was weighed and dissolved in deionized water to prepare a stock of 10 mg/ml. 1 µl of stock was added to the 20 µl DNA sample in TE buffer for digesting unwanted RNA. RNase was from Roche.

29. Running Buffer, 5 X stock solution was prepared by adding 15.14 g of Tris, 93.94 g of glycine, 5 g of SDS to 1000 ml of deionized water. Tris, glycine and SDS were all from Bio-Rad.

30. Sodium acetate was weighed and dissolved in deionized water to prepare the stock at a concentration of 3 M. Stock of sodium acetate was adjusted to pH 5.2. Sodium acetate was used with absolute ethanol for precipitating DNA. Sodium acetate was from Sigma.

31. Sodium Dodccyl Sulphate (SDS) (10 %) was prepared by dissolving 10 g of SDS into 100 ml of deionized water. The mixture was placed in 37-55 ^{o}C hot water bath until the solution was clear. SDS was from Bio-Rad.

32. Sodium nitroprusside (SNP) was prepared freshly in every experiment. SNP was weighed and dissolved in deionized water to obtain the concentration required. SNP was from Sigma.

33. SYBR Green I was used as the dsDNA-specific fluorescent label. 2 µl of SYBR Green I was added to 2 ml run buffer. Both SYBR Green I and the run buffer were from the dsDNA 1000 kit, which was from Bio-Rad.

34. TE buffer contained 5 mM Tris HCl and 20 mM EDTA in deionized water. TE buffer was then adjusted to pH 8.0. TE buffer was used for dissolving DNA for both agarose gel electrophoresis and CE-LIF. Both Tris HCl and EDTA were from GIBCO BRL.

35. Transfer Buffer, 5 X stock solution was prepared by adding 15.14 g Tris and 72.16 g of Glycine to 1000 ml of deionized water. The working solution of transfer buffered

was prepared by adding 200 ml of 5 X stock solution of transfer buffer and 200 ml of methanol to 600 ml of deionized water. Both Tris and glycine were from Bio-Rad. Methanol was from Sigma.

36. Tris 1.0 M was prepared by adding 12.11 g of Tris to 100 ml of deionized water. The pH of Tris was adjusted to 7.4. Tris was from Bio-Rad.
37. Tris-Buffered Saline (TBS), 10 X stock solution was prepared. 12.11 g of Tris and 87.65 g of NaCl was weighed and added to 1000 ml of deionized water. The mixture was stirred and adjusted to pH 7.2. TBS working solution was prepared by one-to-10 dilution. Tris was from Bio-Rad and NaCl was from Sigma.
38. Tris-Buffered Saline with Tween (TBS-T) was prepared by adding 400 ml of 10 X TBS stock solution to 3600 ml deionized water. Two ml of Tween-20 was added to the mixture. Tween-20 was from Fisher Scientific.
39. Typsin (0.05%) for harvesting cells by trypsinization was purchased from Sigma.

2.3 Animals and cell lines

The HRE-H9 rabbit uterine epithelial cell line was obtained from Dr. Lih-Yuh C. Wing (National Cheng Kung University Medical College, Tainan, Taiwan). The HRE-H9 cells were originally developed by immortalizing primary cultures of endometrial epithelial cells, derived from hCG-treated pseudopregnant rabbits, with SV40 temperature-sensitive (ts) mutant virus (Li et al., 1989; Chen et al., 1991). Cisplatin-sensitive (A2780-s) human ovarian epithelial cancer cell lines were gifts from Drs. R. Goal and B. Vanderhyden (Ottawa Regional Cancer Center, Ottawa, Ontario, Canada). Male C57BL/6J mice (30-35g) (4-6 weeks) were an inbred strain imported from Animal Resource Centre of Australia, originating from Jackson Laboratory of the USA.

2.4 Statistical analysis

The data of the amounts of DNA fragmentation were presented as mean ± SEM. The n value indicates the number of individual experiments conducted. The comparisons between groups of data were made by Student paired t-tests, if there were only two treatment groups. If the number of treatment groups were more than two, the comparisons between groups of data were made by one-way analysis of variance, followed by Tukey's test. All analyses were performed using the software GraphPad Prism (Version 3.0, GraphPad Software, Inc., San Diego, California, USA; www.graphpad.com).

Chapter 3: Literature Review

3.1 Morphological Analysis of Physiological Cell Death

Organisms use physiological cell death for a variety of reasons. The term "apoptosis" is often used to describe the physiological death of mammalian cells (Kerr et al., 1972; Wyllie et al., 1980). When individual cells die in a healthy organ, their death is not accompanied by changes that are characteristic of pathological cell deaths. The term "programmed cell death" is commonly used synonymously with apoptosis. Apoptosis is more of a descriptive term, whereas programmed cell death implies that the decision to die was made cell-autonomously, independently of any other cells. Apoptosis is associated with death of isolated cells, rather than contiguous patches or areas of tissue; there is no inflammatory infiltrate; nuclear shrinkage and internucleosomal fragmentation of the DNA occurs relatively early, but changes to the organelles and loss of membrane integrity are relatively late; the dying cells are phagocytosed by neighboring cells.

Necrosis, a pathological cell death, affects many adjoining cells (Searle, et al., 1982). It is characterized by cell swelling, with early loss of plasma-membrane integrity and major changes to the organelles, and the nucleus tends to swell. Necrosis is accompanied by an inflammatory infiltrate of phagocytic cells.

3.1.1 Characteristics of Apoptosis

In apoptosis, phagocytic cells normally sequester antigenically modified apoptotic cells, preventing inflammation and damage to the surrounding tissue (Duvall et al., 1985). Morphological and biological changes during apoptosis include loss of intracellular water and this leads to condensation of the cytoplasm followed by cellular shrinkage and membrane blebbing. There is also chromatin condensation or aggregation and extensive genomic fragmentation in apoptotic cells (Kerr et al., 1972). The condensation starts at the nuclear periphery, and the condensed chromatin often takes on a concave shape resembling a half-moon, horseshoe, or sickle. DNA in condensed chromatin exhibits hyperchromasia, staining strongly with fluorescent or light absorbing dyes. Besides, there is DNA fragmentation into nucleosomal units of about 200 bp fragments. DNA cleavage occurs at internucleosomal linder region, resulting in ladder formation of DNA of 180-200bp or multiples thereof on agarose gel electrophoresis (Compton, 1992). The reason for the ladder type of fragmentation has been attributed to the activities of Ca^{2+}/Mg^{2+}-activated endonucleases.

Furthermore, apoptotic cells may cause formation of apoptotic bodies. The nuclear fragments, together with constituents of the cytoplasm (including organelles), are packaged and enveloped by fragments of the plasma membrane. These structures, called 'apoptotic bodies' are then shed from the dying cell. However, in necrosis, the nucleus undergoes

slow dissolution and there is release of intracellular constituents, including proteolytic enzymes, into the surrounding space. This evokes an inflammatory reaction with local cellular infiltration, vascular damage, edema, and injury to the surrounding tissue. Table 3.1 summarizes the comparative features of necrosis and apoptosis and diagram 3.1 shows the schematic diagram of the morphological changes in necrosis and apoptosis.

Table 3.1: Comparative features of necrosis and apoptosis

Necrosis	Apoptosis
Passive atrophy	**Active, highly regulated degeneration**
Cell response to gross injury or cytotoxic agents	**Intrinsic cellular suicide program**
Loss of cellular homeostasis	No initial major changes
Altered membrane permeability, membrane lysis and rupture	Not seen, at least initially. Preservation of structural integrity and most of plasma membrane functions
Potassium loss; sodium entry; fall in membrane potential	No sodium influx; no change in potassium concentration in cell
Swelling of all cytoplasmic compartments	Cell dehydration, cellular shrinkage, membrane blebbing, Cytosol and nuclei condensation
Destruction of mitochondria and other organelles. Release of intracellular constituents which include proteolytic enzymes	Generally intact organelles, preservation of cellular organelles, including mitochondria and lysosomes
	Protruberances from cell surface separate to form apoptotic bodies
Depletion of cellular energy (ATP)	No depletion of cellular energy
Lowered macromolecular synthesis	Macromolecular synthesis activation is required
Affects tracts of contiguous cells	Affects scattered individual cells
Loose aggregates of chromatin	Highly condensed granular aggregates of chromatin
DNA degradation is not so extensive, product of degradation are heterogenous size	DNA fragmentation, DNA cleaved at internucleosomal linder region

Summarized from Kerr et al., 1972, McConey et al., 1988, Compton, 1992, Kypianou, et al., 1988, Schwartz et al., 1993

Diagram 3.1: Schematic diagram of the morphological changes in necrosis and apoptosis

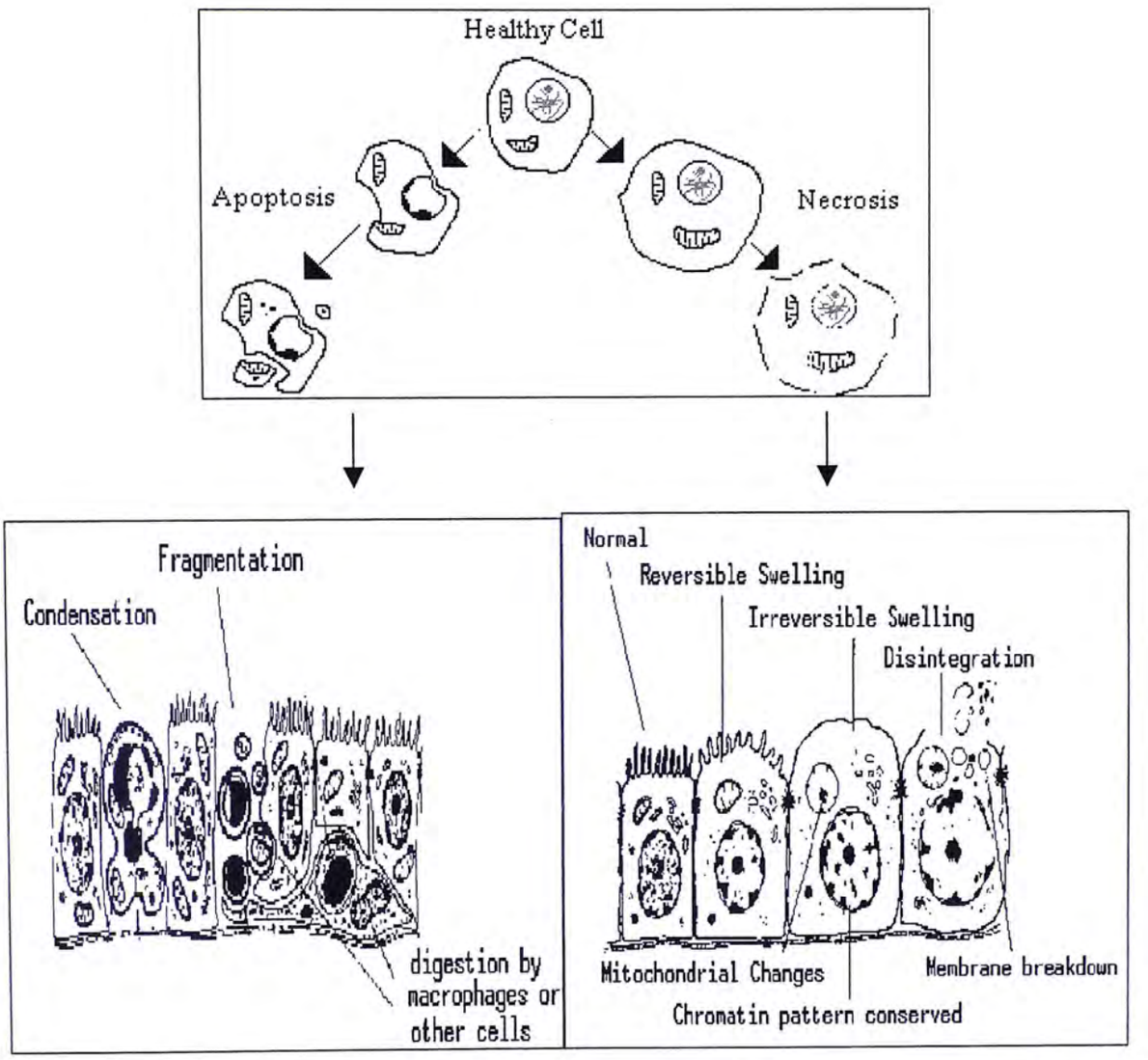


Diagram source from Cell Signaling Technology

3.2 Methods of detecting apoptosis

Many parameters have been used to examine cell death. Numerous methods have been developed to identify apoptotic and necrotic cells and are widely used in various disciplines, in particular in oncology and immunology. The methods are based on changes in cell morphology, plasma membranes structure and transport function, function of organelles, DNA stability to denaturation, and endonucleolytic DNA degradation.

There are a few methods detecting apoptotic cells based on the characteristic of DNA fragmenatation. During DNA fragmentation, DNA is first cleaved into 300kb and 50kb fragments initially (Brown, et al., 1993), then to characteristic 180-200bp internucleosomal fragments. Multiples of the 180-200bp fragments result in a characteristic DNA ladder pattern upon electrophoresis (Wyllie, 1987). Although apoptotic DNA fragmentation is a comon feature of apoptosis in most cells, cell death can still occur in cells lacking the endonucleases needed for apoptotic DNA fragmentation (Nagata. 2000). In these cells, the activation of other pathways can lead to cell death. Nevertheless, apoptotic DNA fragmentation is considered a hallmark of apoptosis in the vast majority of cells. Various techniques, including gel electrophoresis, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) (Kerr et al., 1994; Nagata, 2000) and capillary electrophoresis (Evans et al., 1995; Solis-Recendez et al., 1995; Siles

et al., 1997; Fiscus, 2002; Fiscus et al., 2002) have been used to identify DNA fragmentation in apoptotic cells.

DNA condensation feature can also be used to recognize apoptosis. During apoptosis, nuclear DNA condenses and marginates to the inside of the nuclear membrane (Wyllie et al., 1980). The mechanism for these changes is not known but may be due to cleavage of nuclear lamina and nuclear scaffold attachment factor (Lincz 1998). Flow cytometry and nuclear Hoechst staining utilizing individual cell features of apoptosis to serve as markers for detection and analysis of the proportion of apoptotic cells in the cell population studied.

Another method identifying apoptosis is detection of phosphatidylserine with annexin V. Phosphatidylserine is located on the inner leaflet of the plasma membrane in non-apoptotic cells, and it is translocated to the extracellular side when apoptosis sets in (Adayev et al., 1998). The externalisation to the outer leaflet is an early event in apoptosis. Phosphatidylserine externalisation may be a signal to phagocytes, although this has been shown not to be an absolute requirement for macrophage recognition. Phosphatidylserine acts as a procoagulant, an effect that is reversed when bound to annexin V (Lincz. 1998). Therefore, annexin V labelling can be used to identify apoptotic cells.

Appropriate antibodies to one or more of the cleaved fragments of poly (ADP-ribose) polymerase, which is cleaved by an interleukin-1β-converting enzyme (ICE)-like

protease in apoptotic cells, can also be used (Duriez and Shah, 1997). In situ immunodetection of activated caspase-3, which recognize the p18 subunit of caspase formed during apoptosis can also be used (Srinivasan et al., 1998).

3.3 Molecules controlling apoptosis

The process of apoptosis is mainly controlled by two main groups of molecules, caspases and Bcl-2 family proteins.

3.3.1 Caspases

A novel family of cysteine proteases in the execution of programmed cell death in organisms as diverse as from worms to human has been identified. The name "caspases" was given to the family of cysteine proteases with aspartic acid substrate specificity. The morphological and cellular changes of apoptosis are due, directly or indirectly, to the actions of caspases. Caspases are expressed as zymogens; the active enzymes are thought to be tetramers derived from two pro-caspase molecules, containing the large and small subunits (Cohen, 1997).

At least 14 caspases have been identified in mammals with a uniform nomenclature from caspase-1 to caspase-14 (Alnemri et al., 1996; Nicholson and Thornberry, 1997).

According to their functions in the apoptotic signaling pathway, these caspases are divided into two catagories: the so-called initiator caspases (e.g. caspase-8 and caspase-9) that trigger the activation of the so-called effector or execution caspases (e.g. caspase-3, caspase-6 and caspase-7), that function to interact with other caspases and non-caspase molecules to induce apoptosis by cleaving the cellular substrates. It is well accepted that caspases are not only responsible for degradation of cellular substrates during the end stage of apoptosis, but also are the critical regulators of cell death initiation.

Since caspase activation is the committed step to apoptosis and given the cascade nature of caspase activation, the correct control of caspase activation and thus apoptosis is vital. Thus far, three general mechanisms of caspase activation have been introduced. The simplest way of activating a procaspase is to expose it to another previously activated caspase molecule. The second way of activating procaspase is binding of some ligands. Upon ligand binding, death receptors, such as CD95 (Apo-1/Fas), aggregate and form membrane-bound signaling complexes. These complexes then recruit, through adaptor proteins, several molecules of procaspase-8. This results in localization of high concentration of zymogen. Under these crowded conditions, the low intrinsic protease activity of procaspase-8 is sufficient to allow the various proenzyme molecules to mutually cleave and activate each other. The third way of inducing apoptosis is through association with regulatory cofactors. For example, association with a dedicated protein cofactor, Apaf-1 causes activation of caspase-9.

3.3.2 Bcl-2 family proteins

Besides caspases, the Bcl-2 family of proteins is also involved in the control of apoptosis. Bcl-2 is named after the founding member of the family, which was isolated as a gene product involved in B-cell lymphoma (Tsujimoto et al., 1985). The Bcl-2 family proteins share one or more of 4 Bcl-2 Homology domains, BH1, BH2, BH3 or BH4. Up to now, 15 Bcl-2 family proteins have been identified in mammals; some are pro-apoptotic, whilst some are anti-apoptotic. The Bcl-2 family proteins can be classified into three groups (Adams and Cory, 1998; Antonsson and Martinou, 2000). Group I members contain all four domains and they all possess a C-terminal hydrophobic tail, which localizes the proteins to the outer surface of mitochondria and occasionally of endoplasmic reticulum (ER) with the bulk of the protein facing the cytosol. All group I members are anti-apoptotic. Group II members are pro-apoptotic. Members of this group lack the most N-terminal BH4 domain. Group III members consists of a large and diverse collection of proteins whose only common feature is the presence of the 12-16 amino acid BH3 domain (Kelekar and Thompson, 1998).

The principle mechanism by which Bcl-2 family proteins regulate apoptosis is to control the translocation of cytochrome c from mitochondria to cytosol (Jacobson, 1997; Kelekar and Thompson, 1998; Newton and Strasser, 1998; Reed, 1998). Translocation of the pro-apoptotic Bcl-2 proteins to mitochondria plays the major role in causing

cytochrome c release (Gross et al., 1999; Jacobson, 1997; Kroemer and Reed, 2000; Newton and Strasser, 1998). It is thought that the relative concentrations of pro- and anti-apoptotic Bcl-2 family members provide the control over a cell surviving or undergoing apoptosis. The main protagonists are suggested to be Bax and Bcl-2. Apoptosis can be prevented if the concentration of Bcl-2 is enough to complex with at least half of the Bax. Control of the expression of Bcl-2 family members is actually regulated by other proteins important in the apoptotic process.

3.4 Apoptosis signaling

The recently booming field of cell death research has focused on particular signaling pathways. At least three distinct caspase-signaling pathways exist; one activated through ligand-dependent death receptor oligomerization, the second through mitochondrial disruption, and the third through stress-mediated events involving the endoplasmic reticulum. These pathways also appear to interact to amplify weak apoptotic signals and shorten cellular execution time.

3.4.1 The death receptor-dependent pathway

The death receptor-dependent pathway is mainly present in cytokines-induced apoptosis (Gabuzda and Wang, 2000; Sidoti-de Fraisse et al., 1998). The tumor necrosis

factor receptor (TNFR) family of proteins plays an important role in the mediation of apoptosis or programmed cell death in diverse biological systems (Smith et al., 1994; Gruss and Dower, 1995; Baker and Reddy, 1996). Three members of this family are TNFR1, Fas/Apo-1 (CD95), and death receptor 3 (DR3) (Wsl-1, Apo-3, and TRAMP). Some of these receptors are homologous to each other in extracellular cysteine-rich domains, but also in a cytoplasmic 'death domain' (Itoh and Nagata, 1993; Tartaglia et al., 1993). The death domain is required for protein-protein interaction that ensures down-stream cell death signaling and thus causes induction of apoptosis (Cleveland and Ihle, 1995, Chinnaiyan et al., 1996; Kitson et al., 1996; Marsters et al., 1996; Bodmer et al., 1997). The rapid induction of cell death via the death domain is unique to the TNFR superfamily.

Engagement of the receptors by Fas ligand (Fas-L) or by TNF (or lymphotoxin), respectively, can lead to cell death (Nagata and Golstein, 1995; Baker and Reddy, 1996; Yuan, 1997; Wong and Choi, 1997). These ligands and receptors were the first known members of a multilevel molecular system connecting extracellular signals and the intracellular cell-death cascade. TNFR1 or Fas were suspected to utilize cellular proteins as "downstream messengers of death". To date, several death domain-containing proteins, which associate with either INFR1 or Fas, have been identified and characterized with respect to their ability to induce apoptosis and other downstream signaling events, which are activated in immune responses achieved through ligand binding to this receptor.

On activation by the receptor's ligand, the death domain recruits various proteins that mediate cell death. These proteins, in turn, recruit other proteins via their death domains or death effector domains (DED). The actual destruction of the cell, however, is accomplished by serial activation of a family of proteases referred to as caspases. For example, binding of TNF or FasL to their receptors leads to recruitment of a variety of death domain-bearing adaptor proteins such as Fas associated death domain (FADD). FADD then recruits the most upstream procapases (pro-caspase-8 and -10) to the receptor complex via a distinct protein interaction module DED, resulting in auto-proteolysis of these initiator caspases. The activation of caspase-8/-10 may either initiate a proteolytic cascade of the downstream executioner caspases such as caspase-3 causing demise of the cell (Cryns and Yuan, 1998) or cleave certain pro-apoptotic Bcl-2 family proteins such as Bid, facilitating it translocation to mitochondria to induce cytochrome c release (Budihardjo et al., 1999).

3.4.2 The mitochondria-dependent pathway

Mitochondria are involved in cell death for reasons that go beyond ATP supply. Mitochondria can be considered as the heart of the apoptotic machinery. It is the converging site of many apoptotic signaling pathways which are stimulated by different apoptotic inducers. Mitochondria contain and release proteins that are involved in the

apoptotic cascade, like the caspase activator cytochrome c and apoptosis inducing factor (AIF) (Cai et al., 1998; Reed, 1997; Susin et al., 1998). Physiologically, cytochrome c is one of the electron carriers in the respiratory chain, which is localized in the intermembrane space of the mitochondria. Mitochondrial cytochrome c has been found to have dual functions in controlling both cellular energetic metabolism and apoptosis. In mammalian cells, once cytochrome c is in the cytosol, it will bind to apoptotic protease activating factor (Apaf-1). In the presence of ATP or dATP, the complex of cytochrome c and Apaf-1 recruits and activates pro-caspase-9. The activated caspase-9 then activates other caspases, such as caspase-3, which are responsible for cleaving intracellular proteins, leading to apoptotic DNA fragmentation and eventually cell death (Desagher and Martinou, 2000).

3.4.3 The Endoplasmic Reticulum-dependent pathway

The endoplasmic reticulum (ER) serves several important functions, including post-translational modification, folding and assembly of newly synthesized secretory proteins, and a cellular calcium store. Proper function of the ER is essential to cell survival and any perturbation of its function may induce cellular damage and results in apoptosis. Recent studies indicated that the ER is the organelle that can sense cellular stresses leading to apoptosis and transduce apoptotic signals. These cellular stresses include disruption of ER calcium homeostasis and the accumulation of excess protein in the ER. Uniquely,

caspase-12 acts as an initiator of stress-induced apoptosis through the ER despite its dissimilarity to the 'initiator' caspase-8 and -9 (Nakagawa et al., 2000).

Pro-caspase-12, predominately situated on the cytosolic side of ER membrane, can be activated by an imbalance in ER homeostasis (Nakagawa and Yuan, 2000; Nakagawa et al., 2000). The activated caspase-12 then activates the downstream effector caspases either directly or indirectly (Nakagawa and Yuan, 2000).

3.5 Importance of apoptosis in female reproductive system

3.5.1 Apoptosis in uterine epithelial cells

Both apoptosis and proliferation play essential roles in the homeostasis of renewable tissues. Apoptosis is now recognized as an important event in the development and function of the reproductive system (Gosden and Spear, 1997). Progressive increases in apoptosis occur in uterine epithelial cells during the secretory and menstrual phases of the menstrual cycle in humans (Tabibzadeh, 1995) and the estrous cycle in rats (Sato, et al, 1997). Thus, the apoptosis of uterine epithelial cells is thought to play an important role in the regulation of the menstruation cycle and abnormalities in the regulation of epithelial apoptosis may thus result in dysmenorrhea.

Implantation of an embryo into the wall of the uterus also involves apoptosis of epithelial cells. Uterine epithelial cells surrounding the embryos during implantation undergo apoptosis and this leads to the phagocytosis of the epithelial cells by trophoblast cells in mice and rats (Parr et al., 1987; Kamijo et al., 1998). Therefore, induction of apoptosis of endometrial epithelial cells may represent an essential cellular event for successful implantation. A lack of proper apoptosis of uterine epithelial cells during implantation is thought to be one of the causes of infertility. Mechanisms of apoptosis in endometrial epithelial cells during trophoblast invasion, however, remain to be established (Pampfer and Donnay, 1999).

Another condition associated with abnormal apoptotic responses in uterine epithelial cells is endometriosis. Gebel et al., (1998) showed that spontaneous apoptosis of endometrial tissue is impaired in women with endometriosis. Thus, abnormal growth and impaired sensitivity to spontaneous apoptosis in uterine epithelial cells is thought to cause endometriosis.

Bcl-2 is expressed in all the components of the endometrium but at different phases of the menstrual cycle. Bcl-2 expression is concordant geographically and chronologically with proliferation. It starts in the glandular epithelium at the bottom of functional layer and gradually spreads to all the glands. This expression is most intense in the basalis glands, gradually diminishing in the mid and upper functionalis glands (Tabibzadeh et al., 1995;

Gompel et al., 1994; Otsuki et al., 1994). Bcl-2 expression peaks at the proliferative phase. Three days after ovulation, most epithelial cells with the exception of some residing in the basalis, become bcl-2 negative for the duration of the secretory phase. Bcl-2-negative endometrial cells can become susceptible to signals inducing apoptosis. Apoptosis appears in endometrium simultaneously with the disappearance of bcl-2. Persistent expression of bcl-2 in the epithelial cells residing within the basalis may account for the specific privilege that these cells have to escape apoptosis, allowing these cells to contribute to the repair of the endometrium after menstruation.

3.5.2 Apoptosis in ovarian cancer cells

Ovarian cancer is the most lethal gynaecological cancer in the Western world and ranks fifth among the most common female cancers (Wingo et al., 1995). More than 20, 000 women in North America are diagnosed with ovarian cancer each year and more than 12,000 die from the disease. Among all kinds of human ovarian malignancies, epithelial ovarian tumors, which originate from the simple epithelium covering the surface of the ovary, account for over 85%.

Little is known about the biomolecular processes governing the initiation and spread of ovarian cancer. However, it is well accepted that alterations in normal cell proliferation, including increased cell growth and/or decreased cell death, cause an

imbalance in tissue homeostasis. This may contribute to the initiation and progression of tumor growth (Thompson, 1995). Thus, apoptosis plays an important role in maintaining normal ovarian functions and a delicate balance between cell growth and cell death helps to ensure normal ovarian functions.

Recent research has revealed the presence of several potent endogenous suppressers of apoptosis, the inhibitor of apoptosis proteins (IAPs), in mammalian cells. IAPs were first identified in baculoviruses, where they keep the host cells alive while the viruses replicate (Birnbaum et al., 1994; Crook et al., 1993). The IAP family currently consist of six members: neuronal apoptosis inhibitory protein (NAIP) [Roy et al., 1995], X-linked inhibitor of apoptosis protein (XIAP) (Liston et al., 1996; Uren et al., 1996; Duckett et al., 1996), human inhibitor of apoptosis protein-1 (HIAP-1) (Liston et al., 1996; Uren et al., 1996; Duckett et al., 1996), human inhibitor of apoptosis protein-2 (HIAP-2) (Liston et al., 1996; Uren et al., 1996; Rothe et al., 1995), survivin (Ambrosini et al., 1997) and Livin (Kasof and Gomes, 2001). With the exception of NIAP and survivin, which only have the N-terminal repeats named baculovirus IAP repeats (BIRs; required for biologic function), all the other mammalian IAPs identified to date possess both N-terminal BIRs and a C-terminal RING-zinc finger domain, which is required for protein-protein interaction.

The role of XIAP in the regulation of apoptosis in human ovarian cancer has been examined recently. XIAP is localized in human ovarian carcinomas, in which there are

higher levels of XIAP in proliferative epithelial cancer cells compared to apoptotic epithelial cancer cells (Li et al., 2000; Li et al., 2001a). The pro-apoptotic agent cisplatin, a chemotherapeutic agent commonly used in the treatment of ovarian cancer, has been shown to decrease the expression of XIAP, and this response is thought to contribute to the development of apoptosis caused by cisplatin (Li et al., 2000; Li et al., 2001a). XIAP has been shown to suppress apoptosis via caspase -3 and -7 inhibition (Deverasaux et al., 1997; Takahashi et al., 1998) as well as interfering with the Bax/cytochrome c pathway by inhibiting caspase-9 (Deveraus et al., 1998, 1999). Over-expression of IAPs has been shown to have an anti-apoptotic effect in mammalian cells. For example, over-expression of NIAP, XIAP, HIAP-1 or HIAP-2 protects Chinese hamster ovary (CHO) and RAT-1 cells from apoptosis triggered by menadione (Liston et al., 1996) and over-expression of XIAP or HIAP-2 protects HeLa cells from apoptosis induced by transient transfection of pro-interleukin-1β-converting enzyme (Uren et al., 1996). Furthermore, over-expression of XIAP in human ovarian epithelial cancer cells markedly attenuates cisplatin-induced apopotosis (Asselin et al., 2001; Li et al., 2001a). Therefore, the IAPs appear to play key roles in the regulation of apoptosis in cancer cells.

3.6 Regulation of Apoptosis by Nitric Oxide/cGMP/Protein Kinase G

3.6.1 Regulation of apoptosis by Nitric Oxide

Nitric oxide (NO) is a free-radical molecule generated from L-arginine through the action of NO synthase (NOS). In addition to its role as an endothelium-derived relaxing factor (Moncada et al., 1991), NO also regulates platelet adhesion and aggregation (Radomski et al., 1987; Radomski et al., 1990), leukocyte recruitment and activation (Kubes et al., 1991), and cytokine-induced endothelial cell activation (De catarina et al., 1995). NO is potentially cytotoxic under pathological conditions such as septic shock, inflammation (Brown, 1997), myocardial infarction, and atherosclerosis (Radomski and Salas, 1995).

Despite clear evidence that NO plays an important role in the physiological and pathological processes, there is increasing evidence that NO mediates cell death either via necrosis or via apoptosis. However, effect of NO on apoptosis is contradictory. In some situations, NO activates the transduction pathways leading to apoptosis. However, in other cases, NO protects cells against spontaneous or induced apoptosis. Actually, NO's effect on apoptosis depends on many factors, such as NO concentration, flux and cell types.

NO is known to induce apoptosis in a number of different kinds of cells including activated macrophages of mice (Albina et al., 1993; Sarih et al., 1993), rat vascular smooth muscle cells (Fukuo et al., 1996), and rat neurons (Bonfoco et al., 1995). Moreover, there is evidence that cytokines and a variety of chemical NO donors induce apoptosis in freshly isolated rat islets and clonal pancreatic B-cell lines (Loweth, 1997; Kaneto et a., 1995;

Rabinovitch et al., 1994; Ankarcrona et al., 1994), as well as pulmonary epithelial cells (Janssen et al., 1997). Up to now, the mechanisms by which NO induces apoptosis still remains undefined. It has been proposed that NO toxicity involves its reaction with superoxide to form the strong oxidant peroxynitrite (Beckman, 1991; Beckman et al., 1993: Dawson et al., 1993; Lipton et al., 1993; Troy et al., 1996; Estevez et al., 1998). NO is also known to cause direct damage to DNA by deamination of purines and pyrimidines (Nguyen et al., 1992), in addition to exerting inhibitory effects on metabolic enzymes. Besides, NO also inhibits mitochondrial respiration and induces Ca^{2+} efflux (Balakirev et al., 1997). In addition, NO can also modulate the mitochondrial permeability transition (PT) (Hortelano et al., 1997), a process that is linked to release of apoptogenic factor such as cytochrome c. Furthermore, it has recently been reported that the apoptotic actions of NO in insulin producting HIT-T15 cells are mediated by the sGC/PKG system (Loweth et al., 1997).

Although in many experimental systems NO may favour cell death and even apoptosis, in others NO production may exert a protective effect, either through sGC-dependent or -independent mechanisms (Kolb et al., 2000; Kim et al., 1997). NO has recently emerged as a novel and potent inhibitor of apoptosis. Endogenous NO synthesis or exposure to low level NO donors was first shown to inhibit apoptosis in human B lymphocytes (Mannick et al., 1994), and similar findings have been reported in splenocytes (Genaro et al., 1995), eosinophils (Beauvais et al., 1995), cardiac myocytes (Cheung et al.,

1995), and endothelial cells (Dimmeler et al., 1997).

It has been reported that NO prevents hepatocyte apoptosis initiated by the removal of growth factors or exposure to TNF-α or anti-Fas antibody (Kim et a., 1997). The nitric oxide donors, azide and hydroxylamine were able to inhibit in vitro the programmed cell death of human eosinophils from peripheral blood (Beauvais et al., 1995). Treatment with IL-1β, which stimulated NO production, dose-dependently suppressed the spontaneous onset of apoptosis in ovarian follicles (Chun et al., 1995). NMDA, which can stimulate production of nitric oxide and in turn enhance synthesis of cGMP, enhances the survival of cerebellar granule cells and protects the cells against ethanol-induced cell death by a mechanism that involves the NO/cGMP pathway (Pantazis et al., 1998). Recent studies even showed that iNOS exerts an anti-apoptotic effect on the liver during experimental endotoxemia (Mahidhara and Billiar, 2000; Ou et al., 1997; Li and Billiar, 2000).

Previous studies have demonstrated that NO prevents apoptosis by inhibiting caspase proteolytic activation as well as by directly suppressing caspase activity (Kim et al., 1997; Mannick et al., 1999; Rossig et al., 1999; Tenneti et a., 1997; Melino et al., 1997; Dimmeler et al., 1997). There were several mechanisms proposed to contribute to the anti-apoptotic effect of NO on hepatocytes, including the suppression of activity of multiple caspases by nitrosylation (Kim et al., 1997), inhibition of Bcl-2 cleavage and cytochrome c release (Kim et al., 1998), decreased mitochondrial formation of reactive oxygen

intermediates (Hakoda et al., 1999), and suppression of the induction of pro-apoptotic genes (Zamora et al., 2001). Some of these actions are cGMP-dependent and cGMP analogues suppress caspase activation, loss of mitochondrial membrane potential, and cytochrome c release.

Whether NO protects cells from apoptosis or induces cell death most likely depends on the rate of NO formation and the prominence of caspase-3-like protease activation in the apoptotic process as well as other factors such as the presence of other radicals and antioxidants, or cell type specific factors. Apoptosis is mostly associated with the delivery of NO by chemical donors whereas antiapoptotic effects seem to be related to the endogenous production of NO by NOS.

3.6.2 Regulation of apoptosis by cGMP

In some cell systems, the anti-apoptotic function of NO is mediated, at least partially, by NO-dependent generation of intracellular cGMP. For example, the protection of NO against apoptosis is found to be dependent on production of the secondary messenger cGMP in B-lymphocytes (Genaro et a., 1995), eosinophils (Beauvais et al., 1995), embryonic motor neuron (Estevez et al., 1998), PC12 cells (Kim et al., 1999), and ovarian follicles (Chun et al., 1995). However, it is unknown how cGMP prevents apoptosis signaling and supports survival. cGMP has been shown to up-regulate Bcl-2

expression in splenocytes (Genaro et al., 1995). However, in other cell types, the antiapoptotic mechanism is clearly independent of cGMP (Mannick et a., 1994)

cGMP levels are determined by guanylyl cyclases (GCs), cGMP phosphodiesterases (PDEs) and cGMP export pumps (Patel et al., 1995; Jedlitschky et al., 2000). Soluble guanylyl cyclase (sGC) is a heme-containing enzyme catalyzing the conversion of GTP to cGMP in cells and exists in both particulate and soluble fractions (Waldman and Murad, 1987). sGC activation can be achieved by the binding of NO or compounds such as azide or hydroxylamine to heme. The membrane-bound, particulate GCs (pGCs) are activated by the binding of extracellular natriuretic peptides, guanylin or the heat-stable enterotoxin of E. coli (ST). The effects of sGC on the survival of mammalian cells appear to be different in different cell types and different experimental conditions. For example, activation of sGC has been shown to exert pro-apoptotic effects in some cells types, including cardiomyocytes (Taimor et al., 2000), colon tumor cells (Thompson et al., 2000) and vascular smooth muscle cells (Pollman et al., 1996). However, there were also reports showing protective effects of sGC on the survival of other types of cells, such as neural cells (Garthwaithe and Garthwaithe, 1998; Garthwaite et al., 1995; Estevez et al., 1998; Thippeswamy and Morris, 1997; Fiscus, 2002; Fiscus et al., 2002), leukemia cells (Flamigni et al., 2001) and liver cells (Li et al., 2000).

Cell-membrane-permeant analogues of cGMP have been widely used to elucidate the functional role of cGMP-dependent protein kinase (PKG) in the regulation of biological processes (Sekhar et al., 1992; Butt et al., 1993). PKG, a serine/threonine kinase, is widely expressed in mammalian cells and is activated by cGMP binding. The possible involvement of cGMP and PKG in apoptosis is supported by studies in rat myocytes (Wu et al., 1997), pancreatic β cells (Loweth et al., 1997), and endothelial cells (Suenobu et al., 1999). PKG has recently been shown to play a role in driving exisulind-induced apoptosis of colon tumor cells (Thompson et al., 2000) and NO-induced apoptosis of pancreatic and vascular smooth muscle cells (Kockx and Knaapen, 2000). There is data showing that transfection of PKG increases the sensitivity of vascular smooth muscle cells to apoptosis inducers (Chiche et al., 1998).

3.6.3 Regulation of apoptosis by soluble guanylyl cyclase activator

Different NO donor compounds have been clinically used for over 100 years for the treatment of different vascular and heart diseases, however, these compounds carry the risk of generating tolerance and cross tolerance after prolonged usage (Thadani, 1996), making it necessary to suppress the treatment for several periods (Luke et al., 1987). A potentially physiologically relevant mechanism that sensitises sGC and cGMP has recently been described, and may represent an important new therapeutic intervention. YC-1, [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole], is a chemically synthesized compound that

was originally shown to directly stimulate platelet sGC activity and cGMP production and thereby inhibit platelet aggregation and thrombosis (Ko et al., 1994). The ability of YC-1 to directly activate sGC, to potentiate the stimulatory actions of NO and carbon monoxide (CO) on sGC, and to concurrently cause a persistent elevation of cGMP through inhibition of cGMP breakdown makes it a potentially attractive new therapeutic agent. Since YC-1 is a highly effective vasodilator compound with a prolonged duration of action, the use of YC-1with therapeutic purpose might avoid the problem of tolerance to NO donors. Furthermore, YC-1 boosts the effect of NO and its use might permit the reduction of nitrovasodilator dosage, avoiding the toxic effects due to high NO concentrations (Mülsch et al., 1997). Besides stimulating sGC, YC-1 also inhibits the activity of several PDEs (Ko et al., 1994; Friebe et al., 1998; Galle et al., 1999), thus increasing synthesis of cGMP and decreasing both cGMP and cAMP breakdown in different cell types (Galle et al., 1999). In the present study, we determined the effects of YC-1 on the apoptosis induced by serum deprivation in HRE-H9 cells.

Chapter 4: Apoptotic DNA fragmentation caused by sodium nitroprusside, a nitric oxide donor, in uterine epithelial cells: Ultrasensitive quantitation using the new capillary electrophoresis/laser-induced fluorescence (CE-LIF) technology

4.1 Abstract

Apoptotic cell death of uterine epithelial cells is thought to play an important role in onset of menstruation, successful implantation of embryos and development of endometriosis. Thus, abnormalities in uterine epithelial apoptosis may result in dysmenorrhea, infertility and/or endometriosis. The aim of our study was to determine the toxic, pro-apoptotic effects of sodium nitroprusside (SNP), a NO-releasing agent commonly used in research and clinical treatment, on uterine epithelial cells. These effects were assessed by both the conventional agarose (slab) gel electrophoresis (i.e. DNA laddering) and a new quantitative ultrasensitive CE-LIF technique, recently developed in our laboratory to accurately quantify levels of apoptotic DNA fragmentation. An immortalized cell line (HRE-H9 cells), originally derived from rabbit uterine epithelial cells, and primary cultures of mouse uterine epithelial cells were used as cell culture models. In addition to SNP-induced toxicity, we also determined the effects of serum deprivation and over-crowding culturing condition on DNA fragmentation. When HRE-H9 cells were grown in over-crowded conditions they became apoptotic; i.e. the higher the cell

density, the higher the level of apoptotic DNA fragmentation. Exposure to SNP or serum deprivation induced apoptosis in both HRE-H9 cells and primary cultures of mouse uterine epithelial cells. HRE-H9 cells in serum-free medium for 24 h showed 4.6-fold higher levels of apoptotic DNA fragmentation compared to cells in medium with serum. SNP at 500 and 1000 μM (for 7 h) significantly increased apoptotic DNA fragmentation by 3.0 and 112 fold, respectively. In primary cultures, serum deprivation and SNP (500 μM) caused 21-fold and 12.5-fold increases in apoptotic DNA fragmentation, respectively, but required much longer time (72 h). Our data demonstrated that SNP, serum deprivation and over-crowding conditions all cause elevated apoptotic DNA fragmentation in uterine epithelial cells, as measured by the new quantitative ultrasensitive CE-LIF technique.

4.2 Introduction

Apoptosis is now recognized as an important event in the development and function of the reproductive system (Gosden and Spear, 1997). Progressive increases in apoptosis occur in uterine epithelial cells during the secretory and menstrual phases of the menstrual cycle in humans (Tabibzadeh, 1995) and the estrous cycle in rats (Sato, et al., 1997). The apoptosis of uterine epithelial cells is thought to play an important role in the regulation of the menstruation cycle and abnormalities in the regulation of epithelial apoptosis may thus result in dysmenorrhea.

Implantation of an embryo into the wall of the uterus also involves apoptosis of epithelial cells. Uterine epithelial cells surrounding the embryos during implantation undergo apoptosis and this leads to the phagocytosis of the epithelial cells by trophoblast cells in mice and rats (Parr et al., 1987; Kamijo et al., 1998). Therefore, induction of apoptosis of endometrial epithelial cells may represent an essential cellular event for successful implantation. A lack of proper apoptosis of uterine epithelial cells during implantation is thought to be one of the causes of infertility.

Another condition associated with abnormal apoptotic responses in uterine epithelial cells is endometriosis. Gebel et al., (1998) showed that spontaneous apoptosis of endometrial tissue is impaired in women with endometriosis. Thus, abnormal growth and impaired sensitivity to spontaneous apoptosis in uterine epithelial cells is thought to cause endometriosis.

One of the hallmarks of apoptosis in uterine epithelial cells, like in many types of cells, is the activation of an endonuclease that causes internucleosomal fragmentation of the genomic DNA (Rotello et al., 1989). The conventional technique for assessing apoptotic DNA fragmentation is agarose (slab) gel electrophoresis (i.e. DNA laddering). However, when using this conventional technique, a relatively large amount of DNA and thus a large number of cells, typically 2 to 10 million cells per sample, are needed to obtain clear DNA laddering on agarose gels (Fiscus, 2002; Fiscus et al., 2002). A new technology

has recently emerged using capillary electrophoresis (CE) to separate and identify the apoptotic fragments of DNA in certain types of cells, e.g. thymocytes (Evans et al., 1995), hybridoma cells (Solis-Recendez et al., 1995) and leukemia/lymphoma cells (Siles et al., 1997). Only the study by Evans et al. (1995) had utilized the new ultrasensitive detector, the laser-induced fluorescence detector (LIF), coupled with CE, to observe the apoptotic DNA fragmentation. However, the study did not attempt to quantify the levels of apoptotic DNA fragmentation.

Our laboratory has further developed this CE-LIF technology, using DNA standards to generate standard curves for accurately quantifying the levels of apoptotic DNA fragmentation in two types of cells: epithelial (HRE-H9) cells (Fiscus et al., 2001a) and neural (N1E-115, NG108-15 and PC12) cells (Fiscus et al., 2001b; Fiscus, 2002; Fiscus et al., 2002). With the new CE-LIF technology, apoptotic DNA fragmentation can now be assessed with a sensitivity that is >1,000-fold better than that of the conventional agarose gel electrophoresis technique.

Nitric oxide (NO) can cause either toxic effects, leading to apoptotic and/or necrotic cell death, or protective (anti-apoptotic) effects, depending on the type of cells studied, on the concentration of the NO donor used and on other experimental conditions, such as the levels of superoxide anion, which combines with NO to form the very toxic peroxynitrite (Fiscus, 2002; Fiscus et al., 2002). For example, NO has been shown to

induce apoptosis in the pancreatic B-cell line HIT-T15 (Loweth et al., 1997), endothelial cells (Suenobu et al., 1999) and cardiomyocyte (Shimojo et al., 1999; Arstall et al., 1999). In contrast, NO has anti-apoptotic effects in lymphocytes (Genaro et al., 1995), PC12 cells (Kim et al., 1999) and primary dorsal root ganglion neurons (Thippeswamy et al., 2001).

Data from two recent studies have suggested that NO may have toxic effects in uterine epithelial cells, leading to increased apoptosis. First, isolated slices of human endometrial tissue show increased apoptosis (assessed by TUNEL staining) when incubated *in vitro* with L-arginine, the precursor of NO biosynthesis (Castro et al., 2002). Second, a human endometrial carcinoma cell line (RL95-2 cells), when exposed to sodium nitroprusside (SNP), a NO donor, showed decreased cell survival, assessed by the MTT technique, and increased caspase-3 activity, an indicator of apoptosis (Li et al., 2001b). However, to our knowledge, there have been no previous reports providing an accurate measure of apoptotic DNA fragmentation to quantify the pro-apoptotic effects of NO in uterine epithelial cells. Furthermore, previous reports have not used primary cultures of uterine epithelial cells, presumably because of the limited number of cells that can be obtained in primary cultures and the lack of sensitivity of the previous techniques for determining apoptotic DNA fragmentation.

The purpose of the present study was to use the new quantitative ultrasensitive CE-LIF technique to accurately quantify the effects of the NO donor SNP on apoptotic DNA

fragmentation in uterine epithelial cells, using both a rabbit immortalized epithelial cell line (HRE-H9 cells) and primary cultures of mouse uterine epithelial cells. SNP was selected because it is one of the most commonly used NO donors for both research purposes and clinical treatments (Hardman and Limbird, 2001). In experiments with HRE-H9 cells, we also used the conventional agarose gel electrophoresis technique for comparison. The effects of serum deprivation and overcrowding culturing condition were also investigated to determine their effects on the levels of apoptotic DNA fragmentation in uterine epithelial cells.

4.3 Results

Apoptotic DNA fragmentation induced by overcrowding conditions in HRE-H9 cells

Under normal culturing conditions, the HRE-H9 cells showed a large flat morphology and were attached to the culture dishes firmly. However, as the culturing time increased, the cells began to detach from the culture dishes as observed under the microscope. The HRE-H9 cells were assessed for the presence of apoptotic DNA fragmentation at the different culturing times. The HRE-H9 cells were incubated in medium with 4% fetal bovine serum for 24, 36, 48, 60 and 72 h. Cells in the dish for 48, 60 and 72 h showed increasing internucleosomal DNA fragmentation on agarose gels, which indicated the onset of apoptosis of the cells, starting at about 48 h (Fig. 4.1). As the culturing time

increased, cells grew more densely and the levels of apoptotic DNA fragmentation clearly increased. Thus, overcrowding conditions had induced apoptosis in the HRE-H9 cell.

Apoptotic DNA fragmentation induced by serum deprivation in HRE-H9 cells

Under normal culture conditions, HRE-H9 cells were grown in DMEM with 4 % fetal bovine serum (FBS). Cells grown in medium with 4% FBS showed a large flat morphology and were attached to the culture dishes firmly (at least for the first 48 h). However, when the HRE-H9 cells were incubated in medium without FBS for 24 h, they showed an increased number of cells with rounded morphology and internucleosomal DNA fragmentation (assessed by agarose gels), which indicated the increased presence of apoptosis (Fig. 4.2). The effect of serum deprivation for 24 h was then examined by the new quantitative CE-LIF technique. The amounts of the DNA fragments were clearly larger in the samples from cells incubated in medium without FBS compared with cells incubated in medium with 4% FBS (Fig. 4.3). The peak at 20 min, representing the 540 bp DNA fragment from apoptotic cells, was used to calculate the amount of DNA. The amounts of fragmented DNA were significantly ($P < 0.01$) higher in cells in serum-free medium than in cells in medium with 4% serum (Fig. 4.4). Cells in serum-free medium had a 4.6-fold higher level of apoptotic DNA fragmentation than cells in medium with 4% FBS (Fig. 4.4).

Apoptotic DNA fragmentation induced by the NO donor SNP in HRE-H9 cells

SNP, when added at higher concentrations (e.g. 500 μM and above), caused detachment of some of the HRE-H9 cells from the culture dish, as observed under the microscope. At the concentrations (0, 62.5, 125, 250, 500 and 1000 μM) used, SNP caused detectible fragmentation of DNA only at 500 and 1000 μM, when assessed by agarose gels (Fig. 4.5). The dose-dependent effect of SNP on the induction of apoptosis in HRE-H9 cells was then analyzed using the quantitative CE-LIF technique. SNP dose-dependently increased the levels of apoptotic DNA fragmentation in HRE-H9 cells, in some cases starting at a concentration of 250 μM (Fig. 4.6). The peak at 21 min, representing the 540 bp DNA fragment from apoptotic cells, was used to calculate the amount of DNA. SNP at 250, 500 and 1000 μM caused 1.5-, 3.0- and 112-fold increases in the levels of apoptotic DNA fragmentation, respectively, compared to the controls (Fig. 4.7).

Apoptotic DNA fragmentation induced by serum deprivation or exposure to SNP in primary cultures of mouse uterine epithelial cells

The CE-LIF electropherograms in Figure 4.8 show the effects of serum deprivation and exposure to SNP on apoptotic DNA fragmentation in primary cultures of mouse uterine epithelial cells. The cells were incubated for 72 h in medium with 10% FBS (A),

medium deprived of serum (B) or medium with 10% FBS plus SNP (500 μM) (C). Serum deprivation and SNP increased the levels of apoptotic DNA fragmentation (using the peak representing the 360 bp apoptotic DNA fragment) in the primary uterine epithelial cells by 21 and 12.5 fold, respectively. However, the time of incubation that was needed to induce apoptosis in the primary cultures was much longer than that needed for the HRE-H9 cells. Instead of 24 h in serum-deprivation experiments and 7 h in SNP-exposure experiment needed for induction of apoptosis in HRE-H9 cells, 72 h was needed to induce the apoptotic DNA fragmentation in the primary cultures of mouse uterine epithelial cells.

4.4 Discussion

The present study has used the new quantitative ultrasensitive CE-LIF technology to accurately quantify the toxic, pro-apoptotic effects of the NO donor SNP in uterine epithelial cells. SNP is a pharmaceutical agent that is often used to treat severe hypertension as well as certain heart conditions, such as congestive heart failure and myocardial infarction (Hardman and Limbird, 2001). Furthermore, SNP is one of the most commonly used NO-releasing agents in scientific experiments investigating the pharmacologic or toxic effects of NO. The present study shows the toxic, pro-apoptotic effects of SNP in both an immortalized uterine epithelial cell line, HRE-H9 cells, and primary cultures of mouse uterine epithelial cells. The use of primary cells, isolated from mice, was especially important, because it establishes the capability of studying apoptotic DNA fragmentation in phenotypically-normal cells and provides evidence that future studies with primary cells isolated from genetically-modified mice will be technically possible. In addition to SNP-induced apoptosis, we have also shown that other factors, such as serum deprivation and overcrowded culturing conditions, increase the apoptotic DNA fragmentation in uterine epithelial cells.

Nitric oxide has been reported to have pro-apoptotic effects in various cells, such as macrophages (Albina et al., 1993; Sarih et al., 1993), human articular chondrocytes (Blanco et al., 1995), osteoblasts (Chen et al., 2002), spiral ganglion cells (Pai et al., 1998),

mesangial cells (Sandau et al., 1997), neuroblastoma cells (Yamada et al., 1996) and smooth muscle cells (Nishio et al., 1996). Several mechanisms have been proposed to explain the pro-apoptotic effects of NO. One proposed mechanism is that NO, if present at sufficiently high concentrations, reacts rapidly with superoxide anion to form the highly toxic peroxynitrite ($ONOO^-$) (Estevez et al., 1995; for reviews, see Fiscus, 2002 and Fiscus et al., 2002). Previous reports have shown that peroxynitrite induces both necrosis and apoptosis, depending on its concentration and the conditions of the experiments (Estevez et al., 1995; Salgo et al., 1995; Lin et al., 1995). Both NO and $ONOO^-$ were demonstrated to cause apoptotic DNA fragmentation in vascular smooth muscle cells (O'Connor et al., 1997).

There are also reports showing the opposite effect of NO in the regulation of apoptosis. For example, NO was shown to inhibit apoptosis in gastric mucous cells (Potter and Hanson, 2000) and to protect certain cells from various types of apoptotic stimuli, such as serum deprivation-induced apoptosis in PC12 cells (Kim et al., 1999), lipopolysaccharide-induced apoptosis in pulmonary artery endothelial cells (Ceneviva et al., 1998) and stretch-induced apoptosis in alveolar type II epithelial cells (Edwards et al., 2000). Therefore, NO can act as both a pro-apoptotic agent and an anti-apoptotic agent and seems to have the ability to either initiate or block apoptosis, depending on the type of cells studied, the concentrations of NO used or other conditions of the experiment (Fiscus, 2002; Fiscus et al., 2002).

Both the endothelial form of NO synthase (eNOS) and the inducible form of NOS (iNOS) are expressed in the uterus (Jaing et al., 1996; Telfer et a., 1997; Buhimschi et al., 1996). During the menstruation cycle, the endometrium shows a gradual increase in the expression of iNOS as the cycle progresses from the proliferative phase towards the secretory phase (Tschugguel et al., 1999). In addition, embryos at an early stage of development can generate NO and this NO production seems to be required for normal embryonic development (Gouge et al., 1998). Although the exact biological function of this enhanced NO production is not clearly understood, it has been proposed that NO-induced apoptosis of uterine epithelial cells may be an important step in the progression of the menstrual cycle (Castro et al., 2002). Furthermore, NO-mediated induction of apoptosis in uterine epithelial cells has been proposed to participate in the successful implantation of an embryo (Li et al., 2001b). Apoptosis caused by exposure to a NO donor, like that shown in the present study, may alter the normal regulation of menstruation and embryonic implantation.

The data of the present study show that the addition of exogenous NO, provided by the NO donor SNP, elevates apoptotic DNA fragmentation in both immortalized uterine epithelial cells and primary cultures of mouse epithelial cells, determined by the new quantitative ultrasensitive CE-LIF technology. The present study also shows that overcrowded conditions and serum-deprivation cause apoptotic DNA fragmentation in the uterine epithelial cells. Initially, we had found that the larger the number of HRE-H9 cells

that had been seeded in the culture dishes, the higher the levels of apoptotic DNA fragmentation, measured 24 h after seeding (data not shown). Then, we found that seeding a certain number of cells (5 million cells per 100 mm culture dish), followed by incubation for 24 to 72 h, caused a progressive increase in the levels of apoptosis. This new information will help in establishing the appropriate culturing conditions for the assessments of apoptosis in uterine epithelial cells in future experiments.

Our laboratory had previous utilized the CE-LIF technique to determine if serum deprivation in HRE-H9 cells results in apoptosis. We had shown that serum deprivation causes time-dependent increases in the apoptotic DNA fragmentation in HRE-H9 cells, beginning at 5 h and progressively increasing at 7 and 24 h (Fiscus et al., 2001a). The present study has found a 4.6-fold increase in the 540 bp apoptotic DNA fragments in HRE-H9 cells without serum for 24 h compared to cells with serum. The primary cultures of mouse uterine epithelial cells showed a 21-fold increase in apoptotic DNA fragmentation following serum deprivation. However, the time needed for the induction of apoptosis in the primary cultures was much longer than in the HRE-H9 cell line. For example, 72 h of incubation in serum-free medium was needed to induce apoptosis in the primary uterine epithelial cells, compared with only 5 h in HRE-H9 cells (Fiscus et al., 2001a). To our knowledge, the present study is the first to report the effects of serum deprivation and a NO donor on apoptosis in primary cultures of uterine epithelial cells.

In summary, the present study has used the new ultrasensitive CE-LIF technology to accurately quantify the levels of apoptotic DNA fragmentation caused by the NO donor SNP and by serum deprivation in both rabbit immortalized uterine epithelial cells, HRE-H9 cells, and primary cultures of mouse uterine epithelial cells. Because of the exceptional sensitivity of the CE-LIF technique, only a few thousand cells were needed to measure the apoptotic DNA fragmentation, thus allowing for the first time the accurate quantitation of apoptotic DNA fragmentation in a limited number of cells in primary cultures.

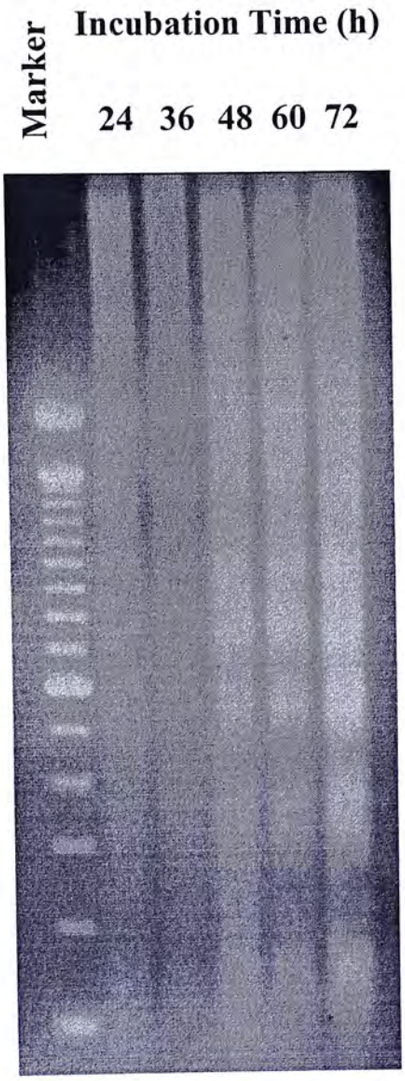


FIG. 4.1. DNA laddering on an agarose gel of DNA samples from HRE-H9 cells grown for 24 to 72 h in medium with serum. Each lane represents a sample from cells grown in a 100 mm culture dish. Samples in all lanes are from cells of the same passage in the same experiment. The image is representative of 3 experiments, all showing similar results.

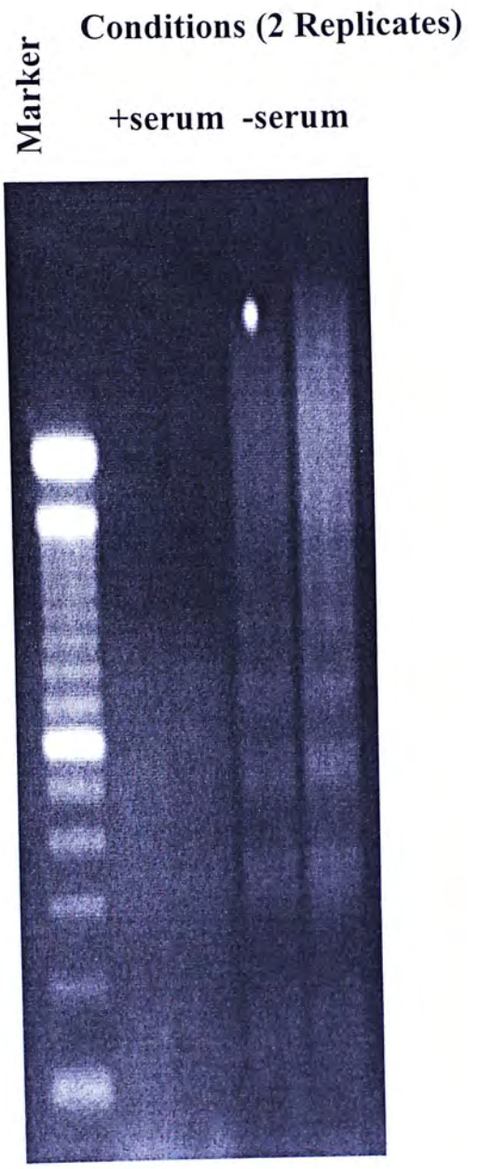


FIG. 4.2. DNA laddering on an agarose gel of DNA samples from HRE-H9 cells with and without serum for 24 h. The second and third lanes represent samples (from two separate experiments) of cells incubated in DMEM culture medium with 4 % serum in 100 mm culture dishes. The fourth and fifth lanes represent samples (from two separate experiments) of cells incubated in DMEM culture medium without serum in 100 mm culture dishes. The image is representative of 5 experiments, all showing similar results.

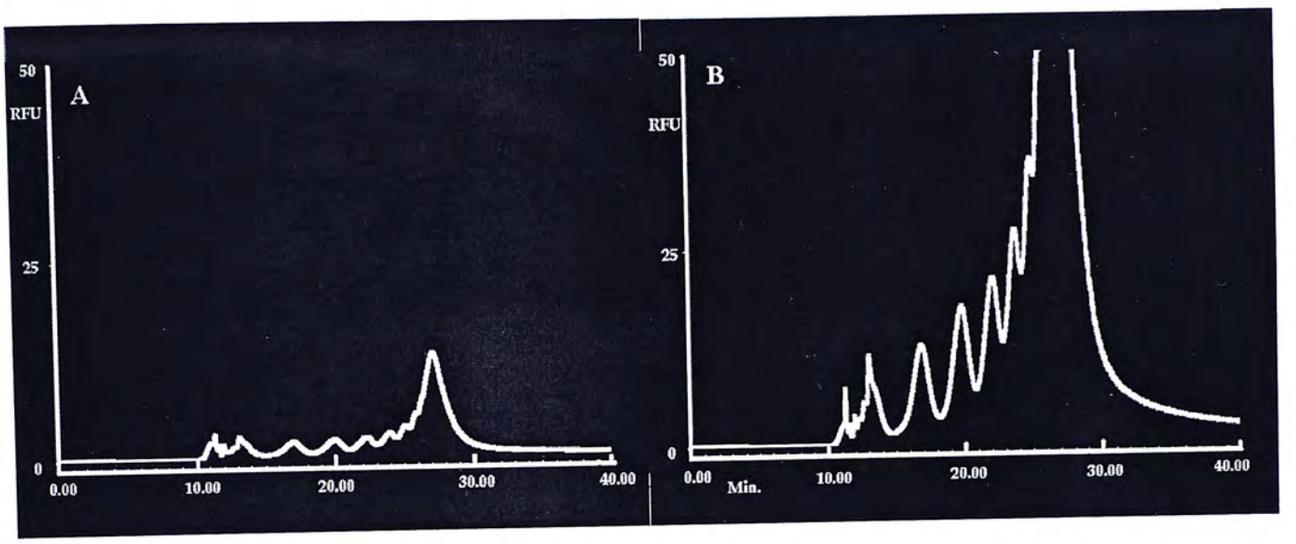


FIG. 4.3. CE-LIF electropherograms of the apoptotic DNA fragments isolated from the HRE-H9 cells with serum (A) and without serum (B) for 24 h. Each electropherogram represents a sample from cells incubated in a well of a 6-well culture plate. Samples in both electropherograms are from cells of the same passage in the same experiment. The electropherograms are representative of 5 experiments, all with similar results. Note the large increase in apoptotic DNA fragmentation in the sample without serum for 24 h.

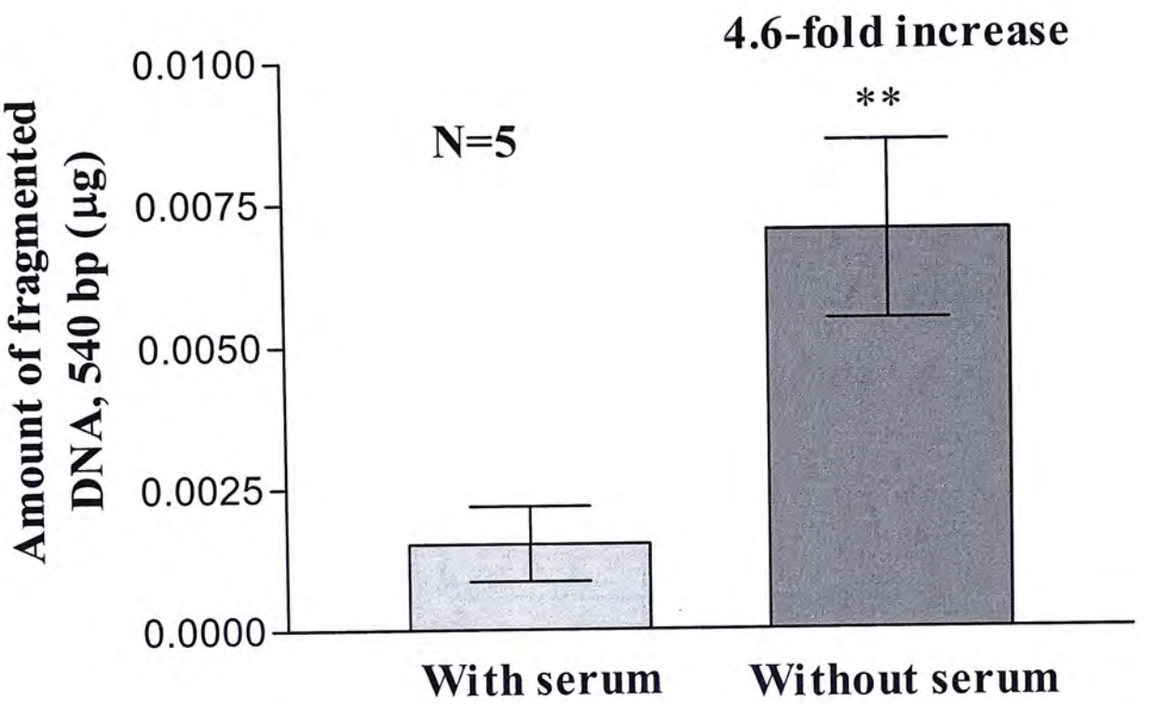


FIG. 4.4. The combined data of 5 experiments showing the amounts of the 540 bp fragment of DNA in samples from HRE-H9 cells with and without serum. Data represent means ± SEM of n=5 samples per group. **P < 0.01 compared to plus-serum control.

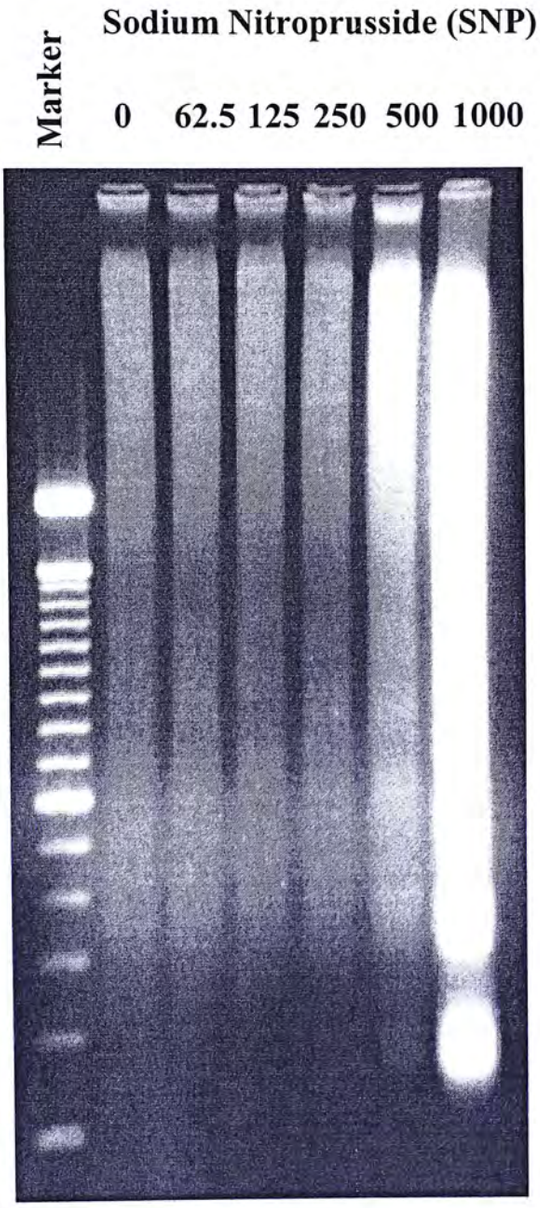


FIG. 4.5. DNA laddering on an agarose gel of DNA samples from HRE-H9 cells exposed to sodium nitroprusside (SNP) at the indicated concentrations. Cells were treated with SNP for 7 h. Each lane represents a sample from cells in a 100 mm culture dish. Samples in all lanes are from cells of the same passage in the same experiment. The image is representative of 5 experiments, all with similar results.

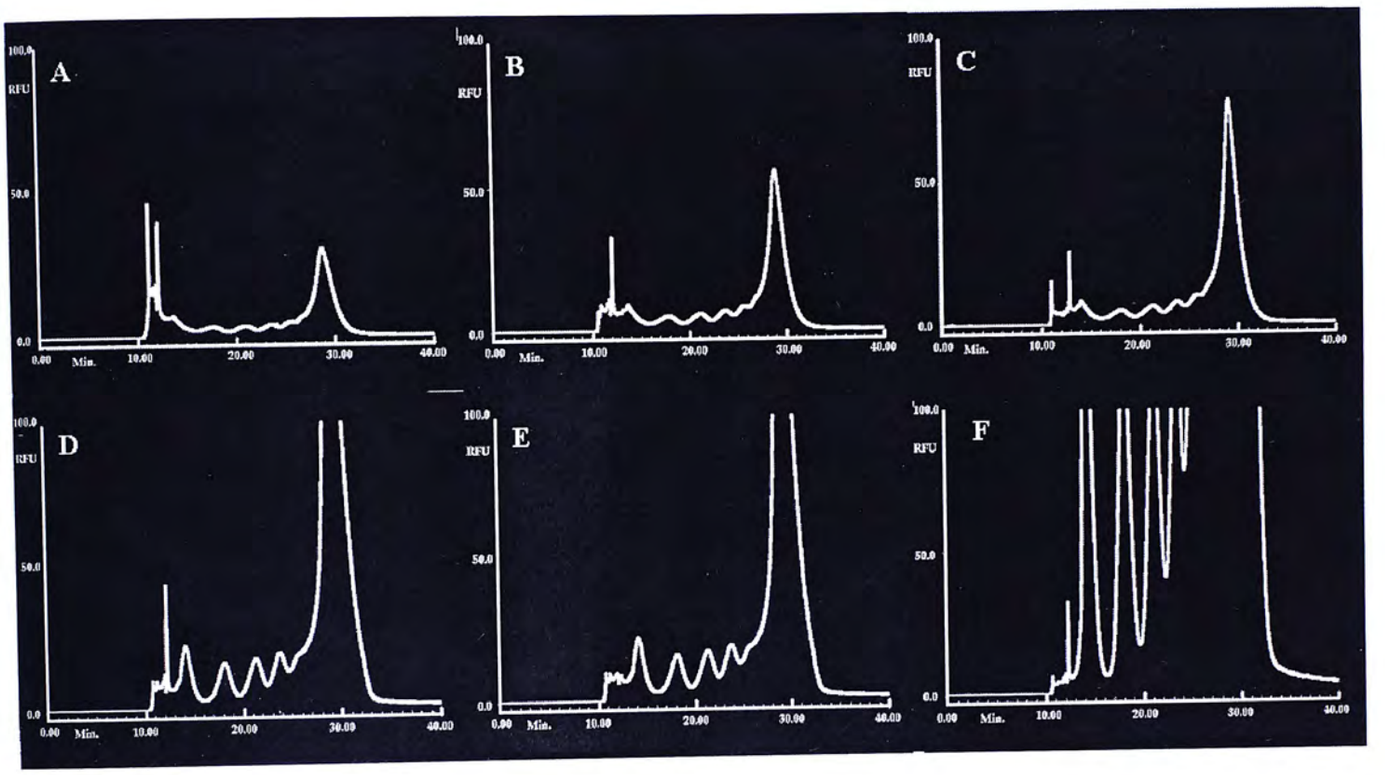


FIG. 4.6. CE-LIF electropherograms of the apoptotic DNA fragments isolated from the HRE-H9 cells exposed to sodium nitroprusside (SNP) at various concentrations (A: 0 μM; B: 62.5 μM; C: 125 μM; D: 250 μM; E: 500 μM; F: 1000 μM). Cells were treated with SNP for 7 h. Each electropherogram represents a sample from cells incubated in a well in a 6-well culture plate. Samples in all electropherograms are from cells of the same passage in the same experiment. The electropherograms are representative of 6 experiments, all with similar results. SNP at 500 and 1000 μM had caused noticeable increases in apoptotic DNA fragmentation in all 6 experiments. However, SNP at 250 μM increased the fragmentation in some, but not all, of the 6 experiments.

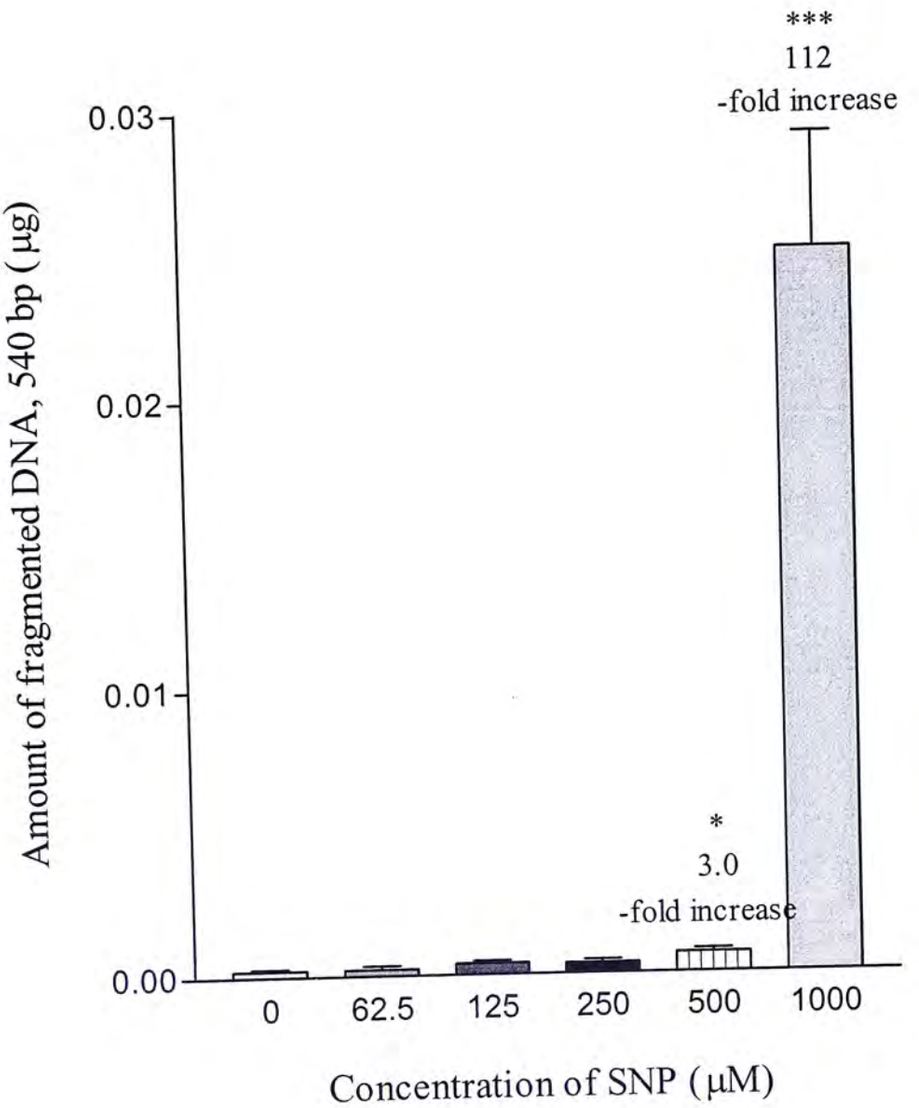


FIG. 4.7. The combined data of 6 experiments, showing the amount of the 540 bp fragment of DNA measured in each of the electropherograms, represented in Fig. 4.6 The data represent the means ± SEM of n=6 samples per group. *P<0.05; ***P<0.001, comparing samples treated with sodium nitroprusside (SNP) with the control group.

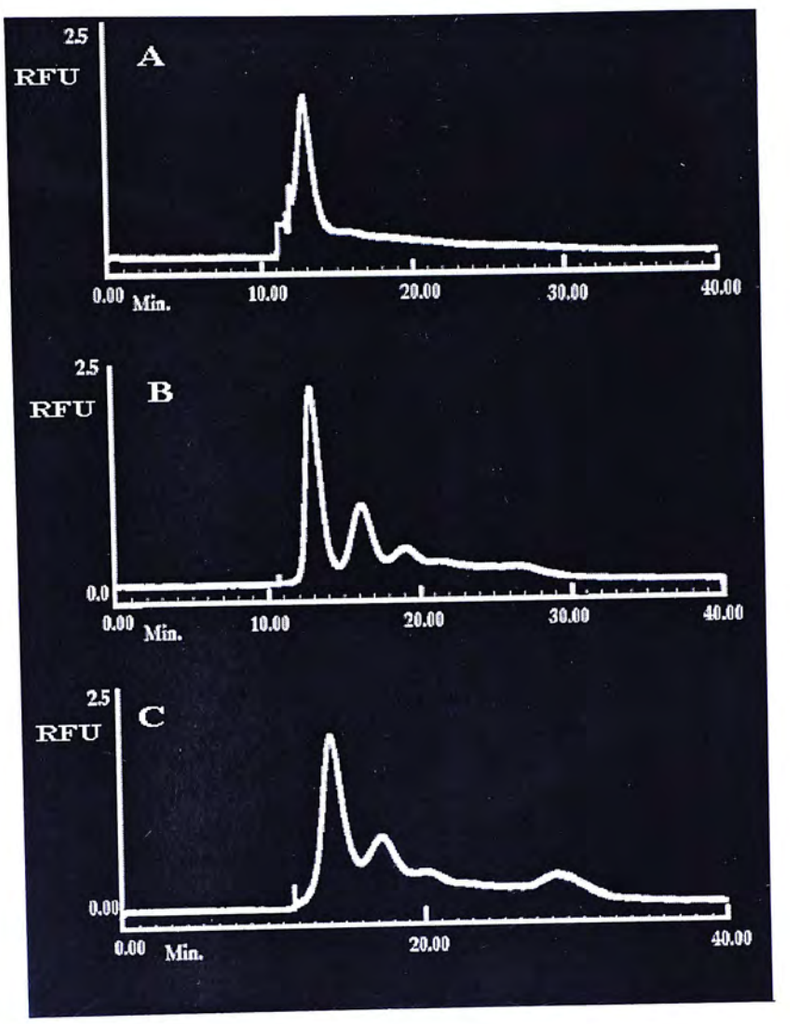


FIG. 4.8. CE-LIF electropherograms of the apoptotic DNA fragments isolated from the primary cultures of mouse uterine epithelial cells, under control (i.e. plus serum) conditions for 72 h (A), without serum for 72 h (B) or exposed to sodium nitroprusside (SNP, 500 μM) in the presence of serum for 72 h (C). Each electropherogram represents a sample from cells incubated in a well of a 6-well culture plate. The electropherograms are representative of 3 experiments, all showing similar results.

Chapter 5: Guanylyl-cyclase inhibitors NS2028 and ODQ and protein kinase G (PKG) inhibitor KT5823 trigger apoptotic DNA fragmentation in an immortalized uterine epithelial cells: anti-apoptotic effects of basal cGMP/PKG

5.1 Abstract

The present study determined involvement of the cGMP/protein kinase G (PKG) signaling pathway in the development of apoptosis in an immortalized uterine epithelial cell line, HRE-H9 cells, using two soluble guanylyl cyclase (sGC) inhibitors, NS2028 and ODQ, and a protein kinase G (PKG) inhibitor, KT5823. Both NS2028 and ODQ significantly ($P<0.05$) increased apoptotic DNA fragmentation in unstressed (with serum present) HRE-H9 cells, measured by a new quantitative ultrasensitive technique using capillary electrophoresis with laser-induced fluorescent detector (CE-LIF), recently developed in our laboratory. NS2028 at 10 and 100 μM caused 2.4- and 3.5-fold increases and ODQ (40 μM) caused 9.9-fold increase in apoptotic DNA fragmentation, suggesting that lowering the basal cGMP levels causes spontaneous activation of a pro-apoptotic pathway. A cell-permeable cGMP analogue, 8-Br-cGMP (100 μM and 1 mM), which directly activates PKG, prevented ODQ-induced apoptosis by 42% and 81%, respectively, indicating that replacement of the lowered cGMP with a direct PKG activator can prevent the apoptosis. Western blot analysis, using an antibody specific for PKG type I (PKG-I),

indicated that HRE-H9 cells express PKG-I at moderate levels. Inhibiting basal PKG activity with KT5823 (100 nM and 1 μM) significantly ($P<0.05$) increased apoptotic DNA fragmentation in HRE-H9 cells by 5.9 and 9.8 fold, respectively. Overall, the data show that inhibitors of basal sGC and PKG activities in unstressed immortalized uterine epithelial cells cause apoptotic DNA fragmentation, suggesting that normal basal levels of cGMP and PKG activity may be necessary to prevent spontaneous development of apoptosis in these cells.

5.2 Introduction

Apoptosis is important to many physiologic/pathgologic processes. Dysregulated apoptosis may contribute to the pathogenesis of a number of diseases, including cancer (Liebermann et al., 1995), autoimmune diseases (Reap et al., 1995), AIDS (acquired immunodeficiency syndrome) (Ameisen, 1992), neurodegenerative disorders (Estevez et al., 1998; Fiscus and Ming, 2000; Fiscus, 2002) and leukemia (Meinhardt et al., 1999; Kolb, 2000). Altering the apoptotic threshold may change the natural progression of some of these diseases. For example, the effectiveness of chemotherapeutic drugs for various cancers may depend on the ability of these agents to induce apoptosis preferentially in the cancer cells (Li et al., 2000b).

In the female reproductive system, apoptosis of the uterine epithelial cells is now recognized to be an important step in the normal onset of menstruation (Tabibzadeh, 1995; Sato, et al, 1997; Tao, 1997; Toki, 1998) as well as the normal implantation of an embryo into the uterine wall during pregnancy (Parr et al., 1987; Kamijo et al., 1998; Galan et al., 2000). Dysregulation of uterine epithelial apoptosis may thus lead to dysmenorrhea and infertility. Abnormal apoptosis of uterine epithelial cells is also thought to contribute to the pathogenesis of endometriosis (Gebel et al., 1998; Garcia-Velasco et al., 2002). Therefore, uterine epithelial apoptosis is an important part of the physiologic/pathologic regulation of the female reproductive system.

Nitric oxide (NO) is known to be involved in the regulation of apoptosis, both as an anti-apoptotic factor and as a pro-apoptotic factor, depending on the type of cells, the concentrations of NO and the experimental conditions (for reviews, see Fiscus, 2002; Fiscus et al., 2002). Previously, NO had been shown to cause toxicity and cell death (both apoptosis and necrosis), notably in the central and peripheral nervous systems, where NO is thought to contribute to the neuronal damage of various neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) (Beckman and Koppenol, 1996), Alzheimer's disease and Parkinson's disease (Fiscus and Ming, 2000; Fiscus, 2002; Fiscus et al., 2002). Recently, NO has been shown to cause apoptosis in a human endometrial carcinoma cell line, RL95-2 cells, and this type of NO-induced apoptosis has been

proposed to be responsible for, at least in part, the apoptosis of uterine epithelial cells that occurs during menstruation and embryonic implantation (Li et al., 2001b).

NO also exerts anti-apoptotic effects in certain cells, either through mechanisms involving activation of soluble guanylate cyclase (sGC) and subsequent elevation of cGMP levels or, in some cells, through mechanisms independent of sGC (Kim et al., 1997; Kolb, 2000; Fiscus, 2002; Fiscus et al., 2002). Endogenous NO synthesis or exposure to low levels of NO donors has now been shown to inhibit apoptosis in a number of different kinds of cells, including B lymphocytes (Mannick et al., 1994; Genaro et al., 1995), eosinophils (Beauvais et al., 1995), ovarian follicles (Chun et al., 1995), endothelial cells (Dimmeler et al., 1997) and rat cerebellar granule cells and cortical cells (Pantazis et al., 1998; Fernandez-Tome et al., 1999). In some of these cells, the anti-apoptotic actions of NO were shown to be dependent on the cGMP elevations (Beauvais et al., 1995; Chun et al., 1995; Genaro et al., 1995), while in other cells, the anti-apoptotic mechanism of NO was independent of cGMP (Mannick et al., 1994).

Previously, data from the laboratory of Professor Fiscus had shown that two natriuretic peptides, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), which activate particulate guanylyl cyclase (pGC) and cause prolonged elevations of cGMP levels, are potent and effective inhibitors of apoptotic cell death caused by serum-deprivation-induced stress in PC12 cells, resulting in a significant prolongation of cell

survival (Fiscus et al., 2001b). Furthermore, 8-Br-cGMP, a cell-permeable analog of cGMP that directly activates protein kinase G (PKG), was also found to inhibit apoptosis in stressed PC12 cells. Thus, cGMP and PKG appear to be intimately involved in the prevention of apoptosis and the prolongation of cell survival, at least in certain types of cells, like PC12 cells. Many other neural cells have also been found to possess a similar anti-apoptotic/pro-survival pathway involving the cGMP/PKG signaling pathway (for reviews, see Fiscus, 2002; Fiscus et al., 2002). Furthermore, in some neural cells, such as N1E-115 and NG108-15 cells, even the basal levels of cGMP and basal activity of PKG appear to be sufficient to cause anti-apoptotic effects, protecting these cells against a spontaneous development of apoptosis (Yuen and Fiscus, 2001; Fiscus, 2002).

To our knowledge, no previous report has shown the involvement of cGMP and PKG in the regulation of apoptosis in uterine epithelial cells. Furthermore, no previous report has focused on the potential involvement of basal cGMP levels and basal PKG activity in protecting epithelial and/or reproductive cells against spontaneous development of apoptosis. The present study was designed to determine if basal cGMP levels and basal PKG activity are involved in the control of apoptosis in uterine epithelial cells, using an immortalized cell line, the HRE-H9 cells. We determined the levels of apoptosis in these cells by quantifying the internucleosomal fragmentation of the genomic DNA, a hallmark of apoptosis in uterine epithelial cells (Fiscus et al., 2001a). The apoptotic DNA fragmentation was measured by a new quantitative ultrasensitive technique using capillary

electrophoresis with laser-induced fluorescence detector (CE-LIF), recently developed in our laboratory (Fiscus et al., 2001a; Fiscus et al., 2001b; Fiscus, 2002; Fiscus et al., 2002).

We used two sGC inhibitors, 4H-8bromo-1, 2,4-oxadiazola (3,4-d) benz (b-1,4) oxazin-1-one (NS2028) and 1H-[1,2,4] oxadiazolo [4,3, -a] quinoxalin-1-one (ODQ), to determine if basal sGC activity and basal levels of cGMP are involved in regulating apoptosis in uterine epithelial cells. The present study shows that both NS2028 and ODQ cause significant increases in the levels of apoptotic DNA fragmentation in the HRE-H9 cells. Some of the ODQ-treated cells were further treated with 8-Br-cGMP to determine if replacement of cGMP with a cGMP analog can relieve the pro-apoptotic effects induced by ODQ. Because of the potential involvement of PKG in uterine epithelial cells, the present study also determined if HRE-H9 cells express PKG, using Western blot analysis, and if basal activity of PKG regulates apoptosis in these cells. The selective PKG inhibitor KT5823 was used to inhibit the intracellular activity of PKG in unstressed HRE-H9 cells. Like the sGC inhibitors NS2028 and ODQ, the PKG inhibitor KT5823 also caused significant increases in the levels of apoptotic DNA fragmentation.

Preliminary data of this project, showing pro-apoptotic effects of ODQ and KT5823 in HRE-H9 cells, have been presented previously in abstract form (Chan and Fiscus, 2002).

5.3 Results

Apoptotic DNA fragmentation induced by the sGC inhibitors NS2028 and ODQ in unstressed HRE-H9 cells

To examine whether basal levels of sGC activity and basal levels of cGMP are involved in regulating the apoptosis of uterine epithelial cells, the HRE-H9 cells were incubated in the presence or absence of NS2028 or ODQ, two potent and selective inhibitors of sGC (Morro et al., 1996; Mulsch et al., 1997; Olesen et al., 1998). All experiments were conducted using unstressed cells (i.e. cells incubated in normal medium containing serum). Exposure of HRE-H9 cells to NS2028 for 24 h caused dose-dependent increases in DNA fragmentation, measured by CE-LIF (Figure 5.1). Figure 5.2 shows that 1 μM NS2028 did not have a significant effect on apoptosis, but NS2028 at 10 and 100 μM significantly increased apoptotic DNA fragmentation in HRE-H9 cells, as compared to the controls ($P<0.05$; $n=3$). NS2028 at 10 and 100 μM caused 2.4- and 3.5-fold increases in DNA fragmentation (Figure 5.2).

Previous studies from our laboratory had shown that ODQ (40 μM, 24 h), by itself, lowers basal levels of cGMP to one-fifth of the normal levels and triggers the onset of apoptosis in two neural cell lines, NG108-15 (Yuen and Fiscus, 2001) and NIE-115 (Fiscus, 2002). In the present study, we found that ODQ (40 μM, 24 h) increased the apoptotic DNA fragmentation in HRE-H9 cells, analyzed by both agarose gels (Figure 5.3) and CE-

LIF (Figure 5.4). ODQ (40 μM) clearly increased the levels of apoptotic DNA fragmentation in both agarose gels and CE-LIF. Figure 5.5 shows the combination of data from 5 experiments using CE-LIF, showing that ODQ (40 μM, 24 h) had significantly increased DNA fragmentation by 9.9 fold in HRE-H9 cells as compared with the controls ($P<0.05$; $n=5$). Therefore, both NS2028 and ODQ can significantly and substantially induce apoptosis in unstressed HRE-H9 cells. The data suggest that cGMP at basal levels may be necessary to prevent spontaneous development of apoptosis in these cells.

The cGMP analogue 8-Br-cGMP prevented the ODQ-induced apoptosis

To test whether the reduction in basal cGMP levels caused by ODQ was responsible for the induction of apoptosis, we determined if replacing the cGMP with a cell-permeable analogue of cGMP, 8-Br-cGMP, could prevent ODQ-induced apoptosis. 8-Br-cGMP at 100 μM and 1 mM substantially decreased the apoptotic DNA fragmentation caused by ODQ, as determined by both agarose gels (Figure 5.6) and CE-LIF (Figure 5.7). Figure 5.8 shows the combined data of 5 experiments using CE-LIF, indicating that 8-Br-cGMP (100 μM and 1 mM) caused 42 % and 81 % inhibition of the ODQ-induced DNA fragmentation, respectively. 8-Br-cGMP (100 μM and 1 mM), by itself, had no significant effect on the levels of apoptosis (Figure 5.8).

Protein expression levels of PKG in HRE-H9 cells.

HRE-H9 cells were analyzed for the presence of PKG protein using Western blot analysis. The primary antibody recognized both the type Iα and type Iβ forms of PKG. Figure 5.9 show the relative levels of PKG-Iα/β expression in HRE-H9 cells, compared to expression levels in three other cells commonly used in our laboratory (Fiscus et al., 2001b; Fiscus, 2002; Fiscus et al., 2002). Whereas PC12 cells showed barely detectable levels of PKG, NG108-15 and N1E-115 cells had high levels of PKG expression. The HRE-H9 cells had intermediate levels of PKG expression. Because some cells, such as vascular smooth muscle cells (Lincoln and Cornwell, 1993) and PC12 cells (present study, Figure 5.9) tend to lose their expression of PKG during passaging, we also tested if the levels of PKG expression in HRE-H9 cells were altered during passaging. Figure 5.9 shows that PKG levels are expressed at moderate levels in all HRE-H9 cells at the three different passages tested. Thus, there was no indication that PKG levels are lost or reduced during passaging in the HRE-H9 cells.

Apoptotic DNA fragmentation induced by the PKG inhibitor KT5823 in unstressed HRE-H9 cells

We suspected that the anti-apoptotic effects of cGMP (at basal levels) were mediated by partial activation of PKG (i.e. basal PKG activity) in HRE-H9 cells. To test

this, we used a potent and highly selective PKG inhibitor, KT5823. Exposure of HRE-H9 cells to KT5823 in a wide range of concentrations for 24 h caused dose-dependent increases in apoptotic DNA fragmentation, measured by CE-LIF (Figure 5.10). Because normal media (with serum) was used, the HRE-H9 cells were under unstressed conditions (except for the exposure to KT5823). While KT5823 at 1 nM and 10 nM did not affect the levels of apoptotic DNA fragmentation, KT5823 at 100 nM and 1 μM (concentrations expected to selectively inhibit PKG; Ki = 234 nM, Calbiochem catalog 2003/2004) substantially increased DNA fragmentation, compared to controls (Figure 5.10). KT5823 at 100 nM and 1 μM caused 5.9- and 9.8-fold increases in the DNA fragmentation levels, respectively (Figure 5.11). The statistical significance of these effects was found to be very high ($P<0.001$), due to the large pro-apoptotic effects of this PKG inhibitor. The data suggest that the low-level activation of PKG that occurs under normal culturing conditions, presumably because of the basal cGMP levels, appears to play an essential role in preventing spontaneous development of apoptosis in unstressed HRE-H9 cells.

5.4 Discussion

The present study demonstrates that two sGC inhbitiors, NS2028 and ODQ, and a PKG inhibitor, KT2823, all cause induction of apoptotic DNA fragmentation in the HRE-H9 immortalized uterine epithelial cells. Because the induction of apoptosis occurred at concentrations of the inhibitors that would be expected to cause specific inhibition of sGC

or PKG, the data suggest that the basal levels of sGC and PKG activity may be essential to prevent spontaneous development of apoptosis in these cells. Furthermore, the present data show that addition of the cGMP analog 8-Br-cGMP, a cell-permeable direct activator of PKG, can prevent the apoptosis induced by ODQ. Thus, the cGMP/PKG signaling pathway appears to play an important role in the regulation of apoptosis in immortalized uterine epithelial cells.

sGC is the main intracellular receptor for NO, whereas the particulate form of this enzyme (pGC) is the receptor for the natriuretic peptides ANP and BNP (Waldman and Murad, 1987; Fiscus, 2002). Elevations in cGMP levels are thought to mediate the anti-apoptotic effects of NO in eosinophils (Beauvais et al., 1995), splenocytes (Genaro et al., 1995), hepatocytes (Kim et al., 1997), rat dorsal root ganglia neurons (Thippeswamy and Morris, 1997), motor neurons (Estevez et al., 1998), pulmonary cells (Janssen et al., 1998), PC12 cells (Kim et al., 1999) and macrophages (Heinloth et al., 2002). Furthermore, our laboratory has shown that ANP and BNP cause anti-apoptotic and survival-prolonging effects in stressed PC12 cells, which is mediated by prolonged elevations of cGMP levels (Fiscus et al., 2001b). The PKG activator 8-Br-cGMP also protected these cells against stress-induced apoptosis, suggesting that activation of PKG plays an important role in mediating the anti-apoptotic/pro-survival effects of cGMP in PC12 cells. Many other neural cells show similar protection against apoptosis when cGMP levels are elevated or

PKG activity is increased (Fiscus, 2002). Thus, activation of the cGMP/PKG pathway appears to promote cell survival in a number of different types of cells.

Very few reports, however, have focused on the potential involvement of basal cGMP levels or basal PKG activity in the regulation of cell survival. In 1988, Garthwaithe and Garthwaite showed that exposure of young rat cerebellar slices to a sGC inhibitor, methylene blue, caused progressive destruction of differentiating neurons, and that co-administration of a cGMP analog protected against this destruction (Garthwaite and Garthwaite, 1988), suggesting basal cGMP may have neuroprotective effects. However, because of the known toxic effects of methylene blue (e.g. generation of superoxide) (Fiscus, 2002), it was not clear whether the destructive effects were caused by inhibition of sGC (and subsequent lowering of cGMP levels) or by the toxic effects of methylene blue. More recent experiments using a specific sGC inhibitor, ODQ, showed reduced survival of rat dorsal root ganglion neurons (Thippeswamy and Morris, 1997) and rat motor neurons (Estevez et al., 1998). Inhibition of sGC with ODQ was also shown to decrease the survival and activate a pro-apoptotic pathway in L1210 leukemia cells (Flamigni et al., 2001). Recently, we have shown that inhibition of sGC with ODQ lowers basal cGMP levels to 1/5 of normal levels and causes the development of apoptosis in two established neural cell lines, N1E-115 and NG108-15 cells (Yuen and Fiscus, 2001; Fiscus, 2002). These data suggest that basal sGC activity and basal cGMP levels are involved in protecting neural and leukemia cells against spontaneous development of apoptosis.

The present study, showing that two sGC inhibitors, NS2028 and ODQ, both cause apoptotic DNA fragmentation in uterine epithelial cells, suggest that basal sGC activity and basal cGMP levels protect these cells against spontaneous development of apoptosis. Unlike other so-called sGC inhibitors (e.g. LY83583 and methylene blue), which are not potent inhibitors of sGC and have many other biological actions, ODQ has been shown to be a potent and selective inhibitor of sGC (Garthwaite et al., 1995). ODQ has been shown to inhibit cGMP elevations induced by a NO donors in human platelets and rat vascular smooth muscle cells (Moro et al., 1996). Our laboratory has recently shown that ODQ, when used as 40 μM, the concentration used in the present study, effectively lowers basal cellular levels of cGMP to 1/5 of normal levels (Yuen and Fiscus, 2001; Fiscus, 2002). NS2028 has been shown to selectively block both basal activity of sGC and NO-induced activition of sGC (Mulsch et al., 1997; Olesen et al., 1998). The present data thus provide additional evidence that basal sGC activity and basal cGMP levels have anti-apoptotic effects, and show, for the first time, that this cellular mechanism may be essential for protecting against spontaneous development of apoptosis in uterine epithelial cells.

Several downstream pathways have been proposed to mediate the anti-apoptotic effects of elevating cGMP levels (above the basal levels), including induction of Bcl-2 mRNA and protein (Beauvais et al., 1995; Genaro et al., 1995), suppression of the gene expression of the pro-apoptotic Bcl-2-binding protein BNIP3 gene (Zamora et al., 2001),

prevention of cytochrome c release from the mitochondria (Kim et al., 1997; Kim et al., 1999) and activation of c-Src and subsequent induction of tyrosine phosphorylation of Bcl-2 (Tejedo et al., 2001). In astrocytes, the anti-apoptotic effects of cGMP elevations have been attributed to inhibition of the mitochondrial permeable transition pore (Takuma et al., 2001). Further experiments with uterine epithelial cells will be needed to determine which signaling pathway is involved in the anti-apoptotic effects of basal sGC activity and basal cGMP levels in these cells.

PKG has been shown to be activated by cGMP both in the test tube (for review, see Lincoln and Cornwell, 1993) and in various intact mammalian tissues exposed to either NO (Fiscus et al., 1983; Fiscus et al., 1984) or the natriuretic peptide ANP (Fiscus et al., 1985; Fiscus and Murad, 1988), suggesting that PKG is the major downstream target protein in the cGMP signal transduction pathway. Recently, the anti-apoptotic effects of both elevated and basal levels of cGMP have been proposed to be mediated by PKG activity in neural cells (for review, see Fiscus, 2002). In the present study, we found that HRE-H9 cells express moderate levels of PKG, intermediate between the levels in other cells showing anti-apoptotic effects of cGMP (i.e. NG108-15 and N1E-115 cells with high PKG levels and PC12 cells with low PKG levels). We also found that the PKG inhibitor KT5823, by itself, causes apoptosis in HRE-H9 cells. Because these pro-apoptotic effects of KT5823 occurred at concentrations (i.e. 100 nM and 1 μM) that would be expected to selectively inhibit PKG, the data suggest that PKG activity under basal conditions (i.e.

without elevation of cGMP levels) is sufficient to provide continuous anti-apoptotic effects in HRE-H9 cells.

In summary, the present study shows that selective inhibitors of sGC or PKG cause apoptosis in HRE-H9 immortalized uterine epithelial cells, suggesting that sGC and PKG activities under basal/unstimulated conditions may be essential to prevent spontaneous development of apoptosis. We found that HRE-H9 cells express PKG at moderate levels, which may be sufficient to provide effective, continuous anti-apoptotic effects (even at basal levels of cGMP), thus promoting the survival of these cells. Because of the importance of uterine epithelial apoptosis in normal menstruation and fertility and in the pathogenesis of endometriosis, the anti-apoptotic effects of the cGMP/PKG pathway describe in the present study may have special importance in the regulation of a number of female reproductive functions.

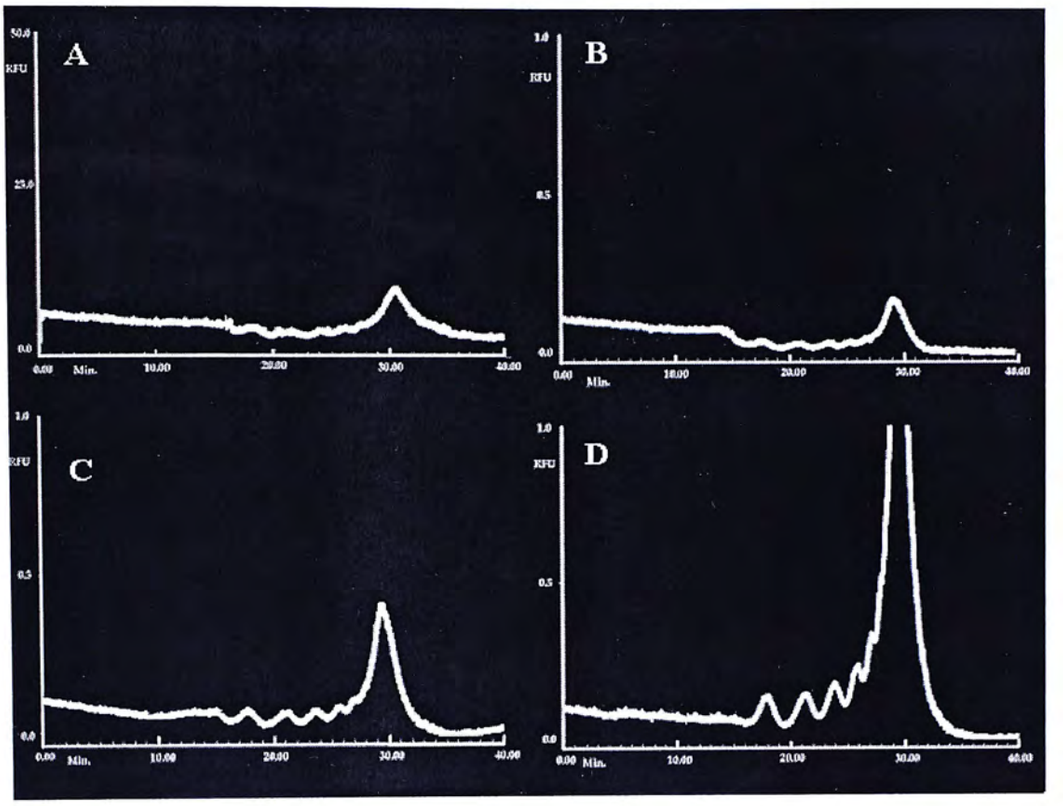


Figure 5.1. CE-LIF electropherograms of the apoptotic DNA fragments isolated from the HRE-H9 cells exposed to NS2028 at 0 μM (control) (A), 1 μM (B), 10 μM (C) and 100 μM (D). The exposure time in each case was 24 h. Each electropherogram represents a sample from cells grown in a well of a 6-well culture plate. Samples in all electropherograms were from cells of the same passage in the same experiment. The electropherogram set is representative of 3 experiments.

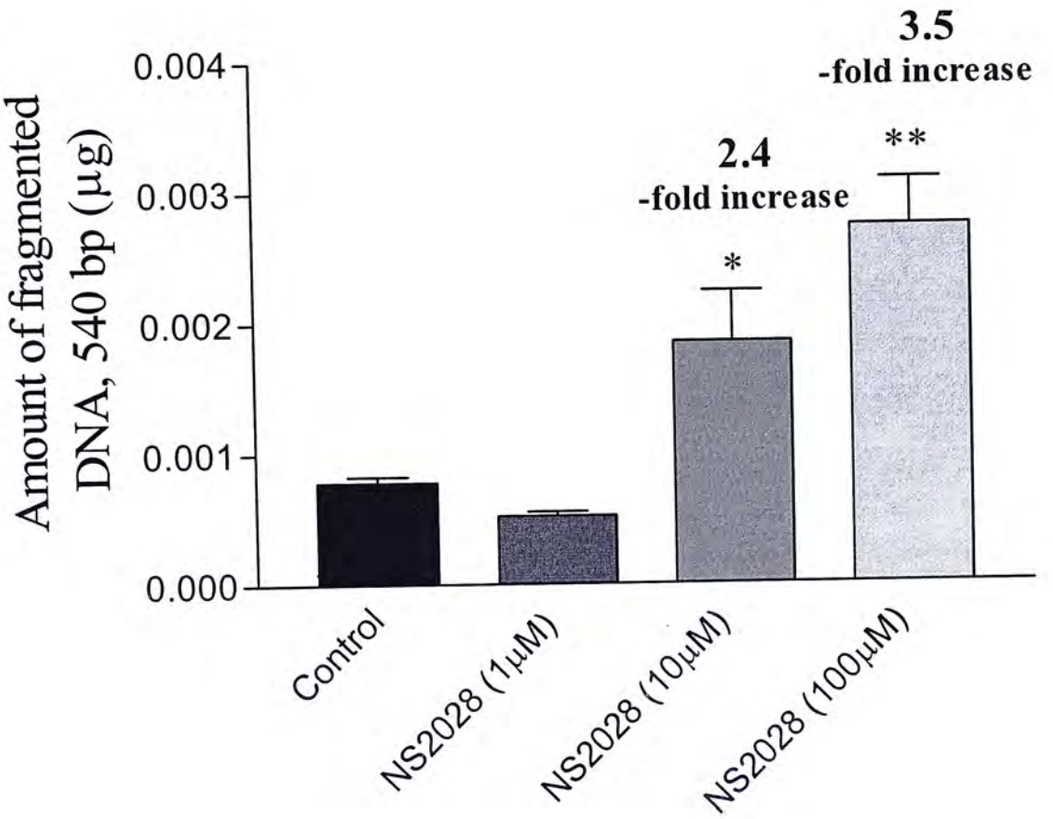


Figure 5.2. The combined data of 3 experiments showing the dose-dependency of the pro-apoptotic effect of NS2028 (24 h). The amounts of the 540 bp DNA fragment were measured in samples from HRE-H9 cells in the different treatment conditions. The data represent the means ± SEM of n=3 samples per group. *$p<0.05$ and **$p < 0.01$ comparing the treatment groups to the control. The number on the top of each bar indicates the fold-increase in the amounts of DNA fragmentation compared to control.

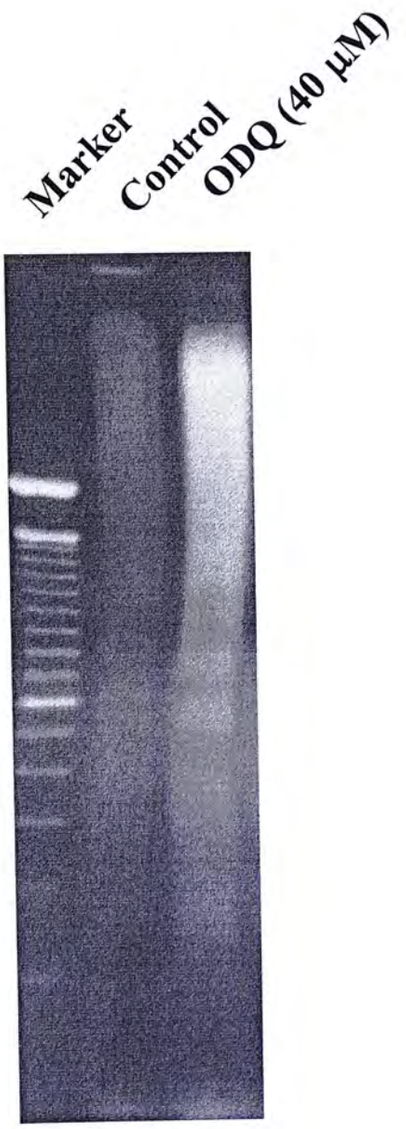


Figure 5.3. DNA laddering on an agarose gel of DNA samples from HRE-H9 cells incubated for 24 h in medium with serum (Control) and medium with serum plus ODQ (40 μM). Each lane represents a sample from cells in a 100 mm culture dish. Samples in all lanes are from cells of the same passage in the same experiment. The image is representative of 3 experiments.

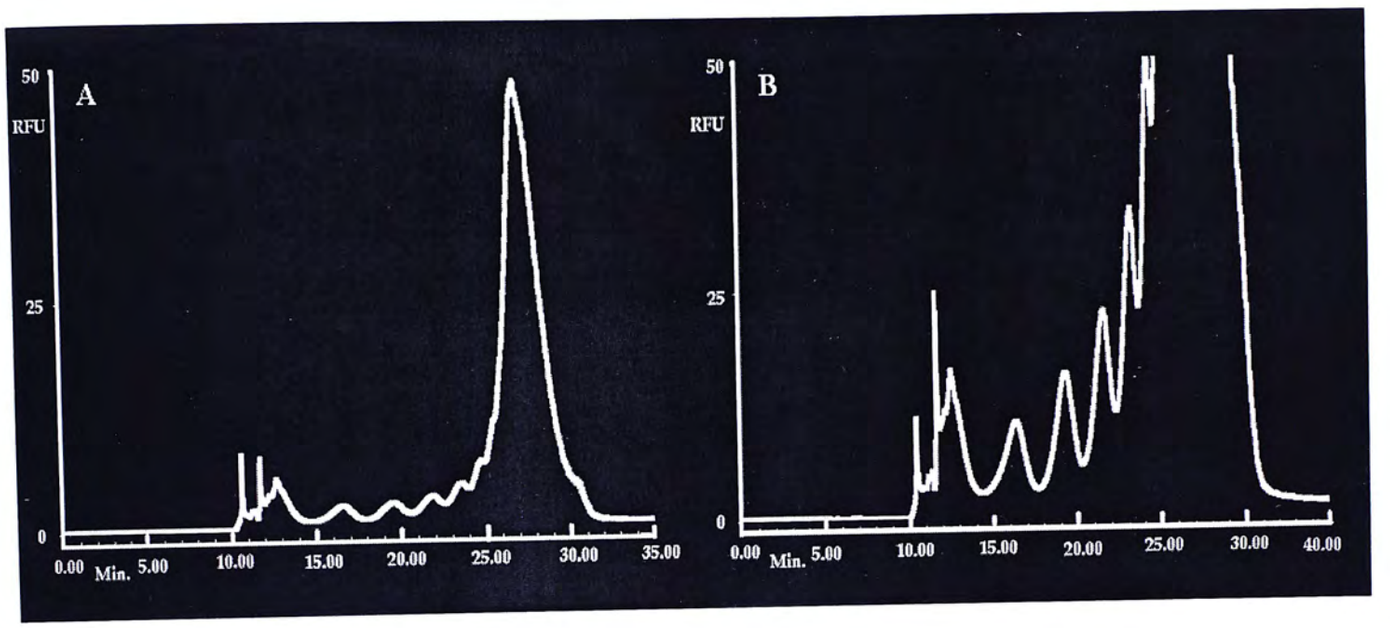


Figure 5.4. CE-LIF electropherograms of the apoptotic DNA fragments isolated from the HRE-H9 cells exposed to ODQ. (A) Control. (B) Cells treated with ODQ (40 μM) for 24 h. Each electropherogram represents a sample from cells in a well of a 6-well culture plate. Samples in all electropherograms are from cells of the same passage in the same experiment. The electropherogram set is representative of 5 experiments.

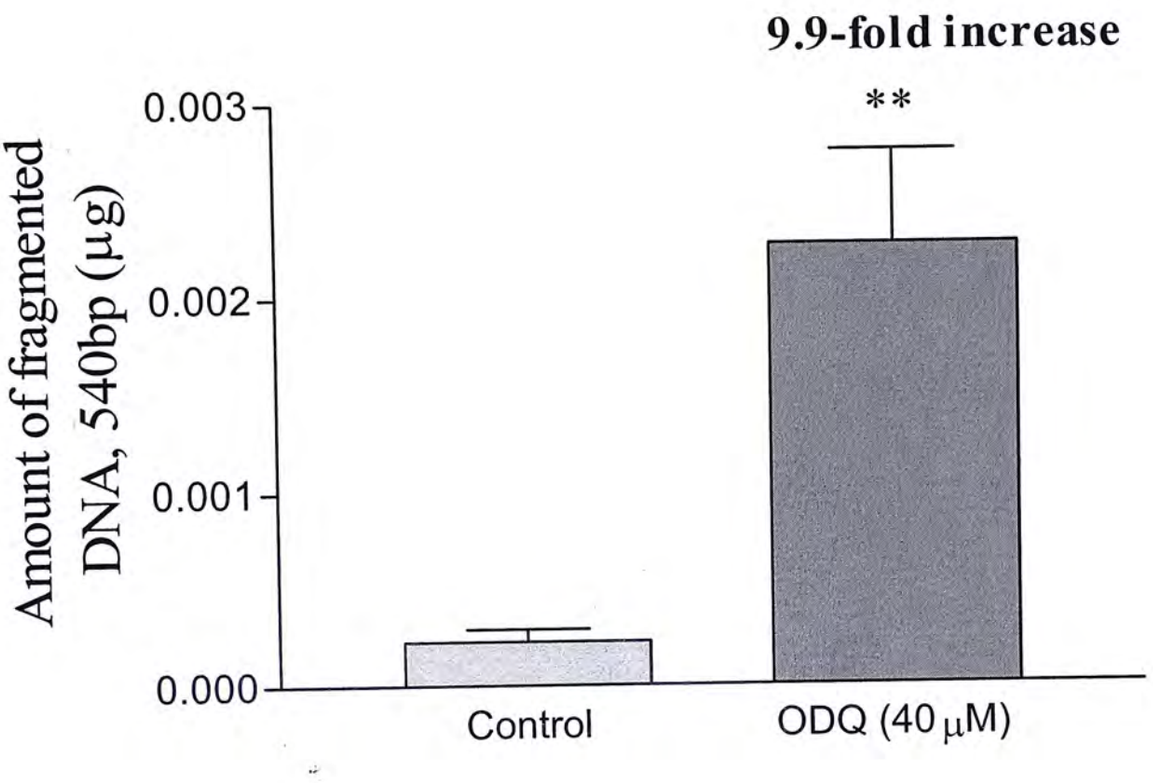


Figure 5.5. The combined data of 5 experiments showing the pro-apoptotic effects of ODQ (24 h). The amount of 540 bp fragmented DNA were measured in samples from HRE-H9 cells in different the treatment conditions. The data represent means ± SEM of n=5 samples per group. **P < 0.01 compared to the control. The number on the top of each bar indicates fold-increase in the amounts of DNA fragmentation compared to the control.

Figure 5.6. DNA laddering on an agarose gel of DNA samples from HRE-H9 cells treated with ODQ or the combination of ODQ and 8-Br-cGMP. Lane 1 represents the control. Lane 2 represents a sample from cells treated with ODQ (40 μM). Lane 3 represents a sample from cells treated with ODQ (40 μM) together with 8-Br-cGMP (100 μM). Lane 4 represents a sample from cells treated with ODQ (40 μM) together with 8-Br-cGMP (1 mM). Each lane represents a sample from cells incubated in a 100 mm culture dish. Samples in all lanes are from cells of the same passage in the same experiment. The image is representative of 3 experiments.

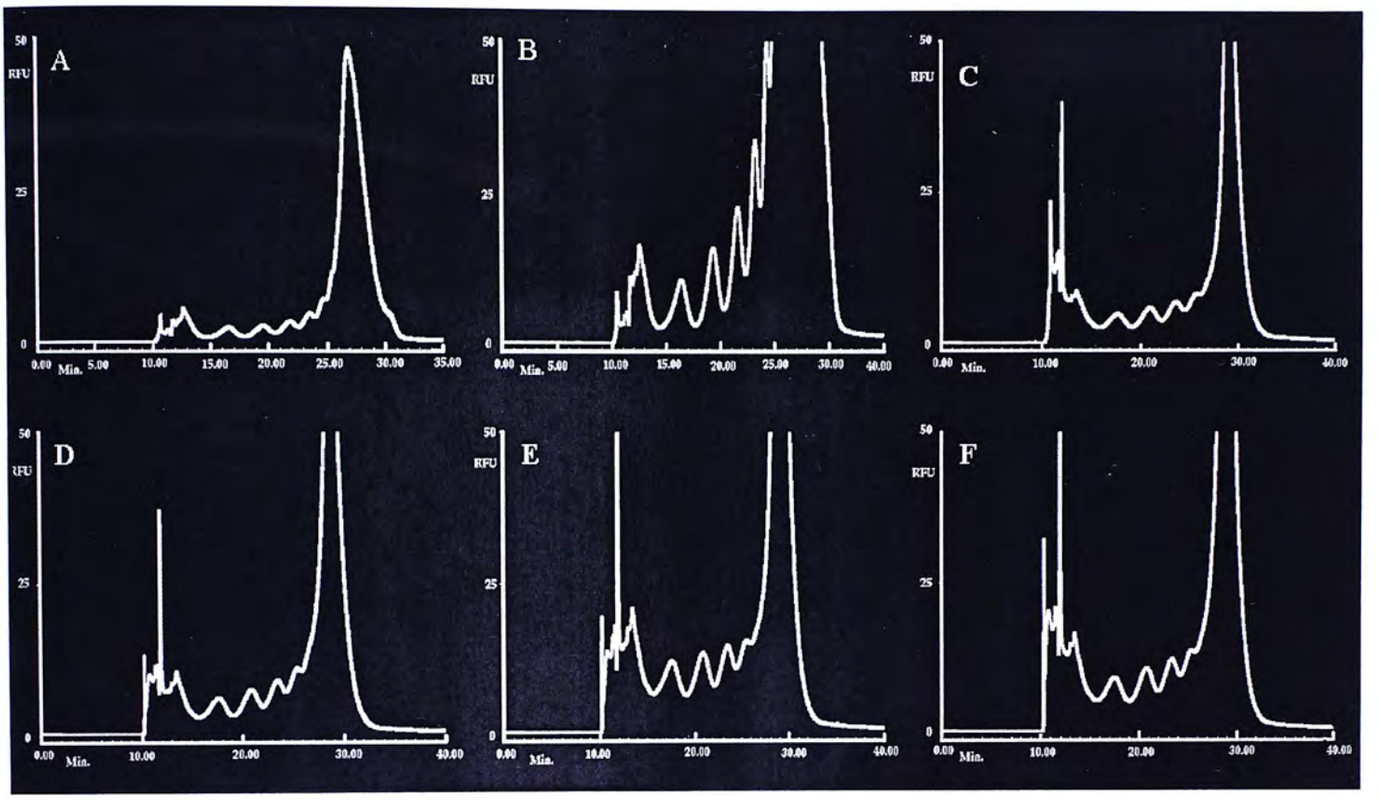


Figure 5.7. CE-LIF electropherograms of the apoptotic DNA fragments isolated from the HRE-H9 cells exposed to ODQ alone or with 8-Br-cGMP: (A) control, (B) cells treated with 40 μM ODQ alone, (C) cells treated with 40 μM ODQ together with 100 μM 8-Br-cGMP, (D) cells treated with 40 μM ODQ together with 1 mM 8-Br-cGMP, (E) cells treated with 100 μM 8-Br-cGMP alone, and (F) cells treated with 1 mM 8-Br-cGMP alone. Cells were all treated for 24 h. Each electropherogram represents a sample from cells incubated in a well of a 6-well culture plate. Samples in all electropherograms were from cells of the same passage in the same experiment. The electropherogram set is representative of 4 experiments.

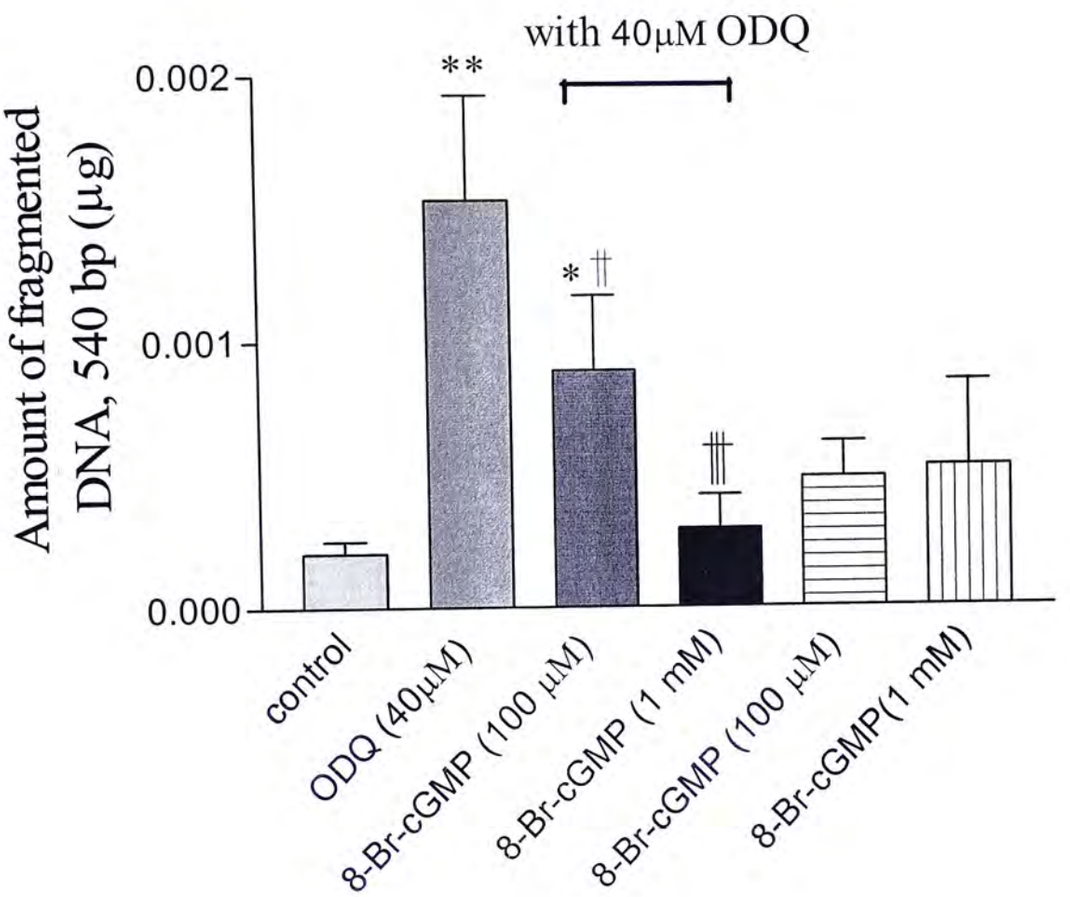


Figure 5.8. The combined data of 4 experiments showing that 8-Br-cGMP can prevent the pro-apoptotic effects of ODQ. The amounts of the 540 bp DNA fragments were measured in samples from HRE-H9 cells in the different treatment conditions. The data represent the means ± SEM of n=4 samples per group. *P<0.05 and **P < 0.01, comparing the treatment groups to the control. ++p<0.01 and +++p<0.001, comparing the treatment groups to the ODQ treated group.

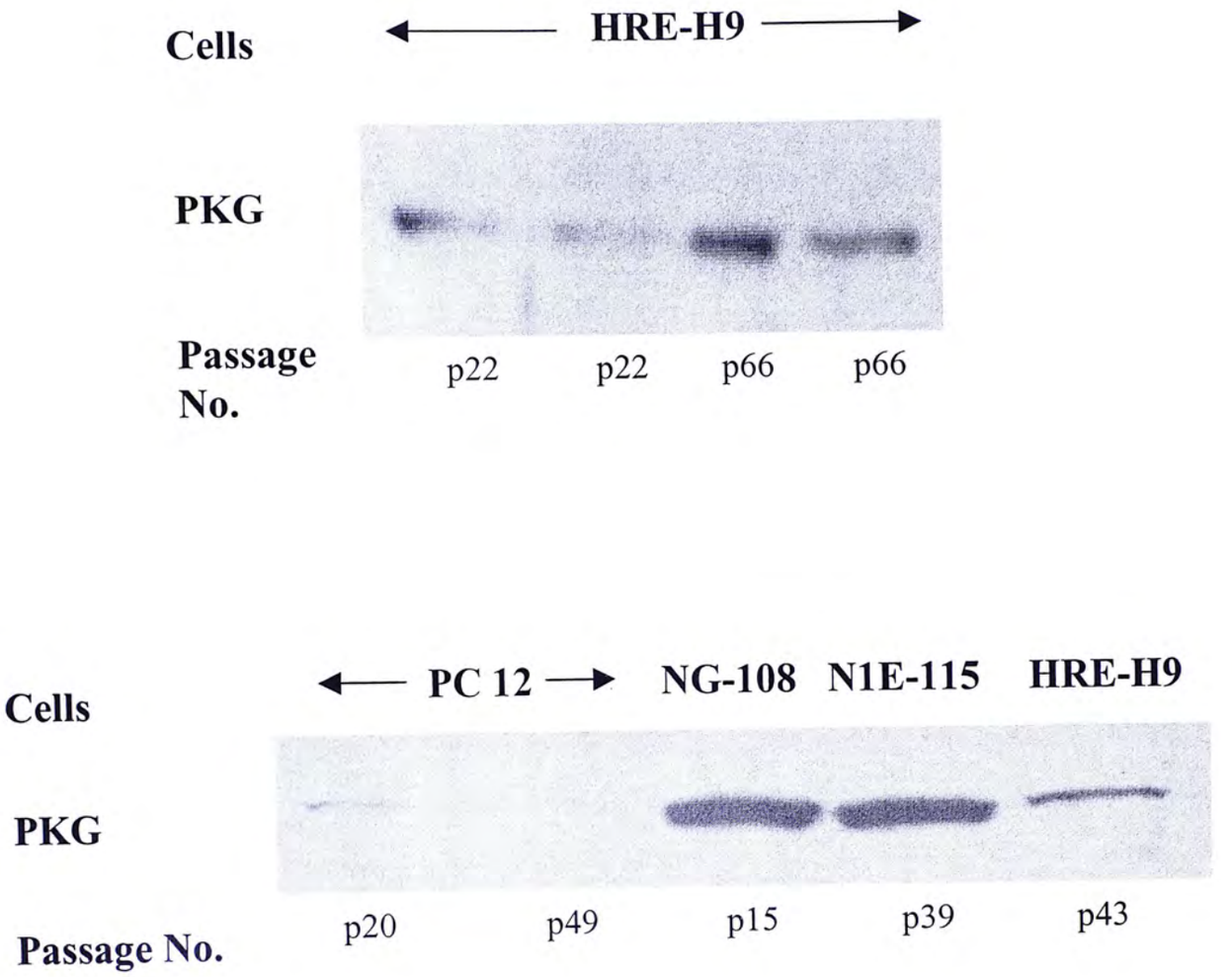


Figure 5.9. Western blot analysis of the PKG expression in HRE-H9 cells. The protein expression levels are compared with 3 other cell lines, known to possess a cGMP/PKG-mediated inhibition of apoptosis.

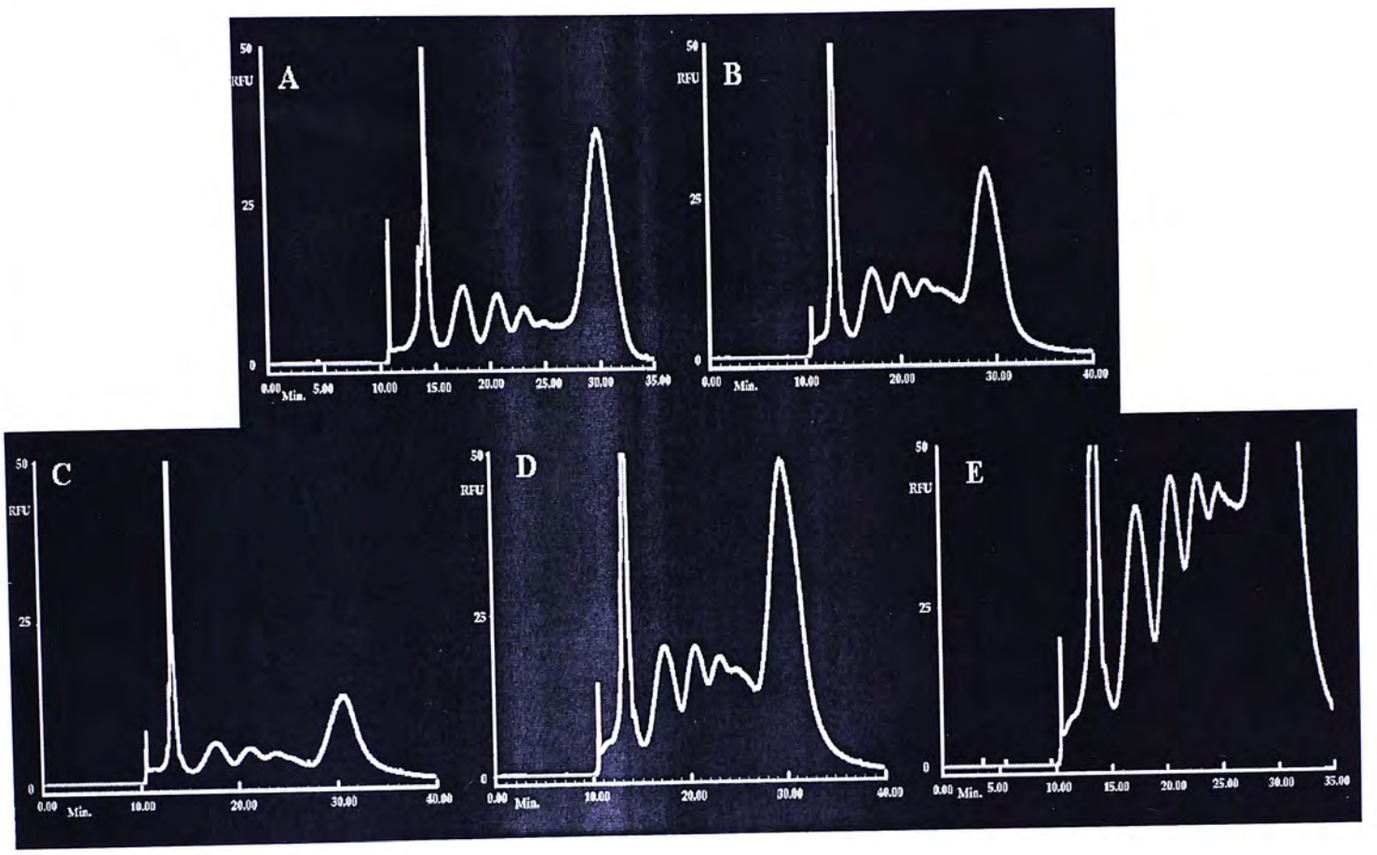


Figure 5.10. CE-LIF electropherograms of the apoptotic DNA fragments isolated from the HRE-H9 cells exposed to KT5823: (A) control, (B) 1 nM KT5823, (C) 10 nM KT5823, (D) 100 nM KT5823, and (E) 1 μM KT5823. Cells were all treated for 24 h. Each electropherogram represents a sample from cells incubated in a well of a 6-well culture plate. Samples in all electropherograms were from cells of the same passage in the same experiment. The electropherogram set is representative of 5 experiments.

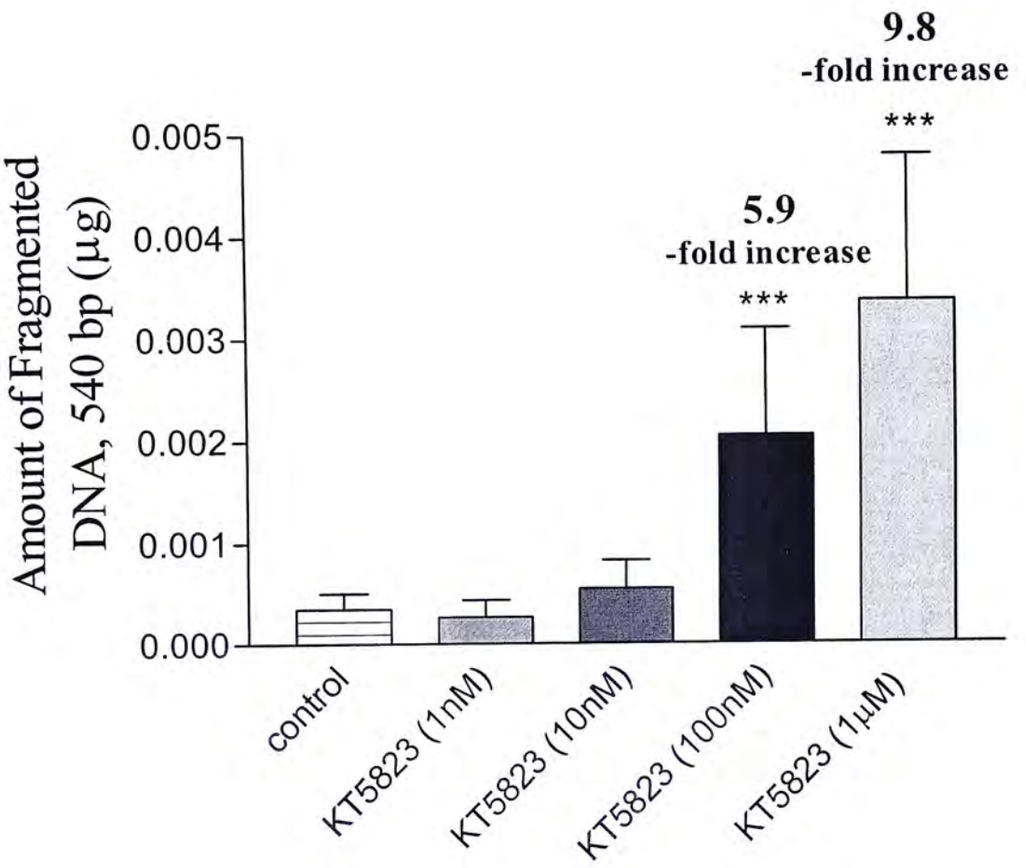


Figure 5.11. The combined data of 5 experiments showing the dose-dependency of the pro-apoptotic effects of KT5823. The amounts of the 540 bp DNA fragments were measured in samples from HRE-H9 cells in the different treatment conditions. The data represent the means ± SEM of n=5 samples per group. ***P < 0.001, comparing the treatment groups to the control. The number on the top of each bar indicates the fold-increase in the amounts of DNA fragmentation compared to the control.

Chapter 6: Direct, prolonged activation of soluble guanylyl cyclase by YC-1 or protein kinase G by cGMP analogs enhances the levels of apoptosis in an immortalized uterine epithelial cell line, HRE-H9 cells

6.1 Abstract

Abnormalities in the regulation of apoptosis of uterine epithelial cells are thought to contribute to dysmenorrhea, infertility and endometriosis. Previous studies have shown that activation of cGMP/protein kinase G (PKG) signaling pathway causes either anti-apoptotic or pro-apoptotic effects, depending of the cell type and/or experimental conditions. Our previous studies with an immortalized uterine epithelial cell line, HRE-H9 cells, showed that basal activity of soluble guanylate cyclase (sGC), responsible for synthesis of most of basal cGMP, and basal activity of PKG were involved in promoting anti-apoptotic effects. However, until now, there have been no reports about anti- or pro-apoptotic effects caused by activating this signaling pathway above basal levels in uterine epithelial cells. The present study determined if activation of sGC or PKG affects serum-deprivation-induced apoptosis in HRE-H9 cells, using a direct, non-nitric oxide activator of sGC, YC-1, and three direct, cell-permeable activators of PKG, 8-Br-cGMP, 8-pCPT-cGMP and Sp-8-Br-PET-cGMPS. YC-1, 8-pCPT-cGMP and Sp-8-Br-PET-cGMPS all enhanced the apoptotic DNA fragmentation induced by serum deprivation, measured by a new quantitative, ultrasensitive technique using capillary electrophoresis with laser-induced fluorescent detector (CE-LIF), recently developed in our laboratory. YC-1 at 100

μM induced 5.4-fold enhancement of apoptotic DNA fragmentation. Sp-8-Br-PET-cGMPS at 10 and 100 μM induced 4.9- and 12.6-fold enhancements of DNA fragmentation. 8-pCPT-cGMP at 1 mM significantly elevated DNA fragmentation by 6.8 fold and 6.2 fold in cells with and without serum, respectively. In contrast, 8-Br-cGMP had no significant effect, presumably because of the more rapid metabolism of this cGMP analog. These data suggest that prolonged elevation of cGMP levels by YC-1 or prolonged activation of PKG by cGMP analogs enhances a pro-apoptotic pathway in serum-deprived immortalized uterine epithelial cells.

6.2 Introduction

Guanylate cylcase (GC) is an enzyme catalyzing the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) in cells and can exist in both particulate and soluble fractions (Waldman and Murad, 1987). The membrane-bound, particulate GCs (pGCs) are activated by the binding of extracellular natriuretic peptides, guanylin or the heat-stable enterotoxin of E. coli (ST), while the soluble form of GC (sGC) is the main intracellular receptor for nitric oxide (NO) (Waldman and Murad, 1987; Garbers, 1992). Cyclic GMP is recognized as an important second messenger that participates in the signal transduction pathways involved in the regulation of various physiological functions, including phototransduction in the rods and cones of the retina, activation and aggregation of platelets and the modulation of intracellular calcium levels in

vascular smooth muscle cells (Lincoln and Cornwell, 1993).

The effects of sGC on the survival of mammalian cells appear to be different in different cell types and different experimental conditions. For example, activation of sGC has been shown to exert pro-apoptotic effects in some cell types, including cardiomyocytes (Taimor et al., 2000), colon tumor cells (Thompson et al., 2000) and vascular smooth muscle cells (Pollman et al., 1996). However, there were also reports showing protective effects of sGC on the survival of other types of cells, such as neural cells (Garthwaite and Garthwaite, 1988; Estevez et al., 1998; Thippeswamy and Morris, 1997; Fiscus, 2002; Fiscus et al., 2002), leukemia cells (Flamigni et al., 2001) and liver cells (Li et al., 2000b).

YC-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole) has been described as a NO-independent, direct activator of sGC (Ko et al., 1994). YC-1 increased cGMP levels by a direct activation of sGC in human platelet homogenate (Wu et al., 1995), adrenomedelluary endothelial cells (Ferrero and Torres, 2001), cardiomyocytes (Taimor et al., 2000) and vascular smooth muscle cells (Mulsch et al., 1997; Galle et al., 1999) in a concentration-dependent manner. In addition, YC-1 has been reported to potentiate both CO- and NO-induced sGC stimulation (Friebe et al., 1996; Friebe et al., 1998). YC-1 was not only shown to activate sGC, but was also shown to stimulate NO synthesis and release through stimulating calcium entry in endothelial cells (Wohlfart et al., 1999). YC-1 also affected cyclic GMP metabolism by inhibiting several phosphodiesterases that cause

cGMP breakdown (Galle et al., 1999). Thus, YC-1 is an effective agent for causing direct activation of sGC and prolonged elevations of cGMP levels in mammalian cells.

Cyclic GMP-dependent protein kinase (protein kinase G or PKG) is thought to be the major downstream target of cGMP within mammalian cells (Fiscus, 1988; Fiscus and Murad, 1988; Lincoln and Cornwell, 1993). Intracellular activation of PKG was first shown in vascular smooth muscle cells following their exposure to the cGMP-elevating agents NO (Fiscus et al., 1983) and atrial natriuretic peptide (ANP) (Fiscus, 1985), and was proposed to mediate NO- and ANP-induced vasorelaxation. More recently, intracellular activation of PKG has been shown in epithelial cells, including bronchial epithelial cells, in which PKG appears to mediate increased frequency of ciliary beating (Wyatt et al., 1998), and intestinal epithelial cells, in which PKG appears to mediate activation of the cystic fibrosis transmembrane conductance regulator Cl channel (Vaadrager et al., 1998). However, the physiological role of PKG is uterine epithelial cells is currently unknown.

Recently, our laboratory has shown that serum-deprivation causes onset of apoptosis and increased levels of DNA fragmentation in an immortalized uterine epithelial cell line, the HRE-H9 cells (Fiscus et al., 2001a). Furthermore, we have shown that inhibition of basal sGC activity using ODQ or inhibition of basal PKG activity using KT5823 causes induction of apoptosis in HRE-H9 cells (Chan and Fiscus, 2002). These

data suggest that the basal activities of sGC and PKG are important for preventing a spontaneous onset of apoptosis in these cells. However, the effects of elevating the intracellular activities of sGC and PKG above basal levels in uterine epithelial cells have not been reported until now.

In the present study, we determined the effects of a direct, NO-independent sGC activator, YC-1, on the apoptosis induced by serum deprivation in HRE-H9 cells. NO was not used as an activator of sGC in the present study because it is known to activate a number of toxic/pro-apoptotic pathways that are independent of the sGC/cGMP pathway (Fiscus and Ming, 2000; Fiscus, 2002; Fiscus et al., 2002,). We further determined the effects of three cGMP analogues, 8-Br-cGMP, 8-pCPT-cGMP, Sp-8-Bromo-PET-cGMPS, all of which are cell-permeable, direct activators of PKG, on the induction of apoptosis in HRE-H9 cells. To measure apoptosis, this study used a novel, quantitative, ultrasensitive technique recently developed in our laboratory for the accurate measurement of apoptotic DNA fragmentation, using capillary electrophoresis with laser-induced fluorescence detector (CE-LIF) (Fiscus et al., 2001a, Fiscus et al., 2001b, Cheuk et al., 2002).

6.3 Results

Enhancement of Apoptosis by YC-1, a Soluble Guanylyl Cyclase (sGC) Activator, in HRE-H9 Cells

To examine whether activation of sGC and, hence, increases in the cGMP levels could modulate the apoptosis of uterine epithelial cells, the HRE-H9 cells were cultured in serum-free medium with and without the sGC activator YC-1. Exposure of HRE-H9 cells to YC-1 for 24 h caused an increase in DNA fragmentation above that of the control cells with serum-deprivation alone, as measured by CE-LIF (Figure 6.1). While YC-1 at 1 μM and 10 μM had no significant effect on cell apoptosis, YC-1 at 100 μM significantly increased apoptotic DNA fragmentation in HRE-H9 cells ($P<0.05$; $n=4$) (Figure 6.2). YC-1 at 100 μM caused 5.4- fold increases in DNA fragmentation above controls.

Effects of the cGMP Analogs 8-Br-cGMP and 8-pCPT-cGMP on Apoptosis in HRE-H9 Cells with and without Serum-deprived Conditions

The cGMP analogs 8-Br-cGMP and 8-pCPT-cGMP are commonly used as potent and selective activators of PKG (Butt et al., 1992). 8-pCPT-cGMP has been shown to be completely resistant to hydrolysis by cGMP-stimulated phosphodiesterase (cGS-PDE, PDE2), cGMP-inhibited phosphodiesterase (cGI-PDE, PDE3), Ca^{2+}-calmodulin-dependent

phosphodiesterase (CaM-PDE, PDE1) and cGMP-specific cGMP-binding phosphodiesterase (cGB-PDE, PDE5). In contrast, 8-Br-cGMP was hydrolysed by these phosphodiesterases, although at a slower rate than cGMP (Butt et al., 1992). Thus, 8-pCPT-cGMP would be predicted to be a better and more persistent activator of PKG, compared to 8-Br-cGMP, because of its better resistance to hydrolysis within mammalian cells.

Because the sGC activator YC-1 had enhanced the apoptosis in HRE-H9 cells and because this pro-apoptotic effect may have been mediated by activation of PKG, we further determined if direct activation of PKG by the cell-permeable cGMP analogs could also enhance the apoptosis. When 8-Br-cGMP at 100 μM and 1 mM was added to the HRE-H9 cells for 24h there appeared to be small increases in the apoptotic DNA fragmentation levels compared to control levels (Figure 6.3). However, these apparent effects of 8-Br-cGMP were not statistically significant at the 5% level (Figure 6.4). In contrast, 8-pCPT-cGMP (1 mM) significantly enhanced the levels of apoptotic DNA fragmentation in HRE-H9 cells in both the presence (Figures 6.5 and 6.6) and absence (Figures 6.7 and 6.8) of serum. 8-pCPT-cGMP (1 mM) elevated the levels of apoptosis in HRE-H9 cells by 6.8 fold (Figure 6.6) in the presence of serum and by 6.2 fold (Figure 6.8) in the absence of serum, compared to the appropriate controls. The data suggested that direct activation of PKG had enhanced the activation of a pro-apoptotic pathway in the HRE-H9 cells. The lack of a consistent response to 8-Br-cGMP was likely due to the hydrolysis of this cGMP analog, thus limiting the length of the PKG activation.

We recognized that a potential technical problem with the use of both 8-Br-cGMP and 8-pCPT-cGMP was that they are also potent activators of the cyclic nucleotide-gated (CNG) cation channels (Wei et al., 1998), which could potentially contribute to the induction of apoptosis because of the influx of Ca^{2+} through the CNG channels. Therefore, to avoid this complication, further experiments were conducted using a more recently developed cGMP analog, Sp-8-Br-PET-cGMPS, that is known to activate PKG (Butt et al., 1995) and inhibit (not activate) the CNG channels (Wei et al., 1996).

Enhancement of Apoptosis by the PKG Activator/CNG Channel Inhibitor Sp-8-Br-PET-cGMPS in Serum-deprived HRE-H9 Cells

Exposure of HRE-H9 cells to Sp-8-Br-PET-cGMPS (1 - 100 μM) for 24 h caused dose-dependent enhancements in the apoptotic DNA fragmentation caused by serum deprivation, as measured by CE-LIF (Figure 6.9). Sp-8-Br-PET-cGMPS at 10 and 100 μM significantly increased the apoptotic DNA fragmentation by 4.9 and 12.6 fold, respectively, as compared with the controls ($P<0.05$; $n=5$) (Figure 6.10).

6.4 Discusion

Previously, data from our laboratory and many other laboratories had shown that the cGMP/PKG signaling pathway plays an important role in protecting mammalian neural

cells against the development of apoptosis induced by serum deprivation and several other forms of cellular stress (for reviews, see Fiscus, 2002, Fiscus et al., 2002). However, the role of the cGMP/PKG pathway in the regulation of apoptosis in uterine epithelial cells has not been established. Our laboratory has shown that serum deprivation causes apoptosis, indicated by increased levels of apoptotic DNA fragmentation, in HRE-H9 uterine epithelial cells (Fiscus et al., 2001a). Furthermore, we have shown that basal levels of cGMP and basal activity of PKG protected these cells against spontaneous development of apoptosis (Chan and Fiscus, 2002), similar to the protective role of basal cGMP/PKG in neural cells (Fiscus, 2002). However, until now it has not been reported whether activation of the cGMP/PKG pathway (above the basal levels) could further protect uterine epithelial cells against the development of apoptosis.

The present study shows that addition of direct activators of sGC or PKG, which would activate the cGMP/PKG pathway above the basal levels, does not protect the HRE-H9 cells against apoptosis induced by serum deprivation. Instead, addition of the sGC activator YC-1 or the PKG activators 8-pCPT-cGMP and Sp-8-Bromo-PET-cGMPS significantly enhanced the apoptotic DNA fragmentation induced by serum deprivation. Thus, unlike the protective effects of basal cGMP/PKG activity, the activation of the cGMP/PKG pathway above basal levels appears to promote, rather than inhibit, apoptosis in these immortalized uterine epithelial cells.

Similar pro-apoptotic effects caused by activation of sGC or PKG have previously been shown in vascular smooth muscle cells (Pollman et al., 1996), cardiac myocytes (Taimor et al., 2000) and colon tumor cells (Thompson et al., 2000). In the case of colon tumor cells, the induction of apoptosis by activation of PKG appeared to involve PKG-mediated phosphorylation of an oncogenic protein, β-catenin, which then results in ubiquitination and subsequent proteolysis of β-catenin (Liu et al., 2001) as well as PKG-mediated phosphorylation of MEKK1 with subsequent activation of the pro-apoptotic JNK1 pathway (Soh et al., 2001). Further experiments with uterine epithelial cells will be needed to determine if the pro-apoptotic effects of YC-1, 8-pCPT-cGMP and Sp-8-Br-PET-cGMPS also involve PKG-mediated phosphorylation of β-catenin and MEKK1.

In the present study, we found that YC-1 (100 μM) significantly enhances serum-deprivation-induced apoptotic DNA fragmentation, quantified by a new ultrasensitive quantitative technique using the new CE-LIF technology (Fiscus et al., 2001a, Fiscus et al., 2001b, Cheuk et al., 2002; Fiscus, 2002, Fiscus et al., 2002). Previously, YC-1 (75 μM) was shown to induce apoptosis in cardiomyocytes via the cGMP signaling pathway (Taimor et al., 2000). Furthermore, YC-1 treatment of colon tumor cells was shown to inhibit growth and induce apoptosis at doses that activate guanylate cyclase (Thompson et al., 2000). However, the effect of YC-1 in the regulation of apoptosis appears to be different in different types of cells. For example, YC-1 exerted anti-apoptotic effects in leukemia cells (Flamigni et al., 2001). Furthermore, we have recently found that YC-1

protects against apoptosis in a neural cell line, NG108-15 cells (unpublished data). Thus, the effects of YC-1 on apoptosis appear to depend on the type of cells.

The present study shows that 8-cPCT-cGMP, but not 8-Br-cGMP, significantly increases the levels of apoptotic DNA fragmentation in HRE-H9 cells. Because 8-Br-cGMP, but not 8-pCPT-cGMP, is metabolized by phosphodiesterases (Butt et al., 1992), the lack of significant pro-apoptotic effects by 8-Br-cGMP is presumably due to the more rapid metabolism, and thus shorter action, of 8-Br-cGMP. 8-pCPT-cGMP has also been shown to induce apoptosis in cardiac myocytes (Taimor et al., 2000), similar to the responses we have found in HRE-H9 uterine epithelial cells.

In addition to activating PKG (Butt et al., 1995), 8-pCPT-cGMP has also been shown to activate CNG cation channels (Wei et al., 1998). Thus, the induction of apoptosis by 8-pCPT-cGMP in our study with HRE-H9 cells may have been due to activation of either PKG or CNG channels. The activation of CNG channel would be expected to cause influx of Ca^{2+}, which could induce a pro-apoptotic response. Therefore, in order to discriminate between the two pathways in their potential involvement in the induction of apoptosis by 8-pCPT-cGMP in HRE-H9 cells, we further investigated the effect of another PKG activator, Sp-8-Bromo-PET-cGMPS, which is a CNG channel blocker rather than activator (Wei et al., 1996). We found that Sp-8-Bromo-PET-cGMPS dose-dependently enhanced the apoptosis induced by serum deprivation in HRE-H9 cells, causing 4.9- and

12.6-fold increases in the levels of apoptotic DNA fragmentation at 10 and 100 μM, respectively. Thus, the enhancement of serum-deprivation-induced apoptosis caused by the cGMP analogs appears to be mediated by PKG activation, rather than activation of CNG channels.

The present data further illustrate the differences in the pro- and anti-apoptotic effects of the sGC/cGMP/PKG signaling pathway in different cell types. Whereas the present study demonstrates the pro-apoptotic effects of sGC and PKG activators in HRE-H9 uterine epithelial cells, many other studies have shown anti-apoptotic effects of sGC and PKG activators in a number of different types of neural cells (Fiscus et al., 2001b; Fiscus, 2002; Fiscus et al., 2002). However, this difference may not be completely dependent on the cell type, but may also be due to the relative levels of activation of sGC and PKG within the different types of cells. For example, our previous experiments with HRE-H9 cells had shown that basal activities of sGC and PKG protected against a spontaneous onset of apoptosis (Chan and Fiscus, 2002). The present study shows that when the sGC and PKG activities are stimulated above the basal levels, the effect of sGC/cGMP/PKG pathway seems to switch from an anti-apoptotic to a pro-apoptotic effect. Thus, in uterine epithelial cells the sGC/cGMP/PKG signaling pathway appears to play a dual role, i.e. protecting against apoptosis when stimulated at low (basal) levels, but inducing apoptosis when stimulated at higher levels. This pro-apoptotic effect of the highly-activated sGC/cGMP/PKG pathway may participate in the increased levels of

apoptosis of uterine epithelial cells that occur during menstruation and implantation of an embryo within the uterine wall, because both events involve an exaggerated production of NO that highly activates the sGC/cGMP/PKG pathway (Gouge et al., 1998, Tschugguel et al., 1999).

A recent publication that profiles the expression levels of 14 different protein kinases during the neoplastic transformation of human ovarian surface epithelial cells has identified PKG as the one protein kinase showing the most dramatic change in expression levels during the transformation of these cells from a normal phenotype to a tumorigenic phenotype (Wong et al., 2001). PKG expression levels were relatively high in normal ovarian epithelial cells, considerably lower in SV40-immortalized non-tumorigenic ovarian epithelial cells and undetectable in SV40-immortalized ovarian epithelial cells that had been further transformed into tumorigenic cells by the transfection of cadherin. Also, three ovarian cancer cell lines were found to have undetectable levels of PKG. Although further experiments are needed to precisely determine the role of PKG in these cells, it is tempting to speculate that the dramatic loss of PKG is an important part of the transformation and tumorigenic process in epithelial cells. Based on the data of the present study, showing pro-apoptotic effects of PKG activation in epithelial cells, it would be predicted that a loss of PKG expression during transformation may increase the cells resistance to the induction of apoptosis, thus contributing to the enhanced survival of the transformed epithelial cells.

In conclusion, the activities of sGC and PKG and the levels of cGMP in uterine epithelial cells are likely to be strictly regulated and to participate in both anti-apoptotic and pro-apoptotic pathways regulating cell survival. Although basal activities of sGC and PKG have anti-apoptotic effects, the elevation of the sGC and PKG activities above basal levels appears to switch the anti-apoptotic effect to a pro-apoptotic effect. Future studies will be needed to determine the precise downstream targets of cGMP and PKG mediating these effects in uterine epithelial cells. Because the cGMP/PKG pathway is activated by NO, the NO-mediated apoptosis of uterine epithelial cells known to occur during menstruation and implantation of the embryo may be mediated, at least in part, via the pro-apoptotic effects of cGMP/PKG activation.

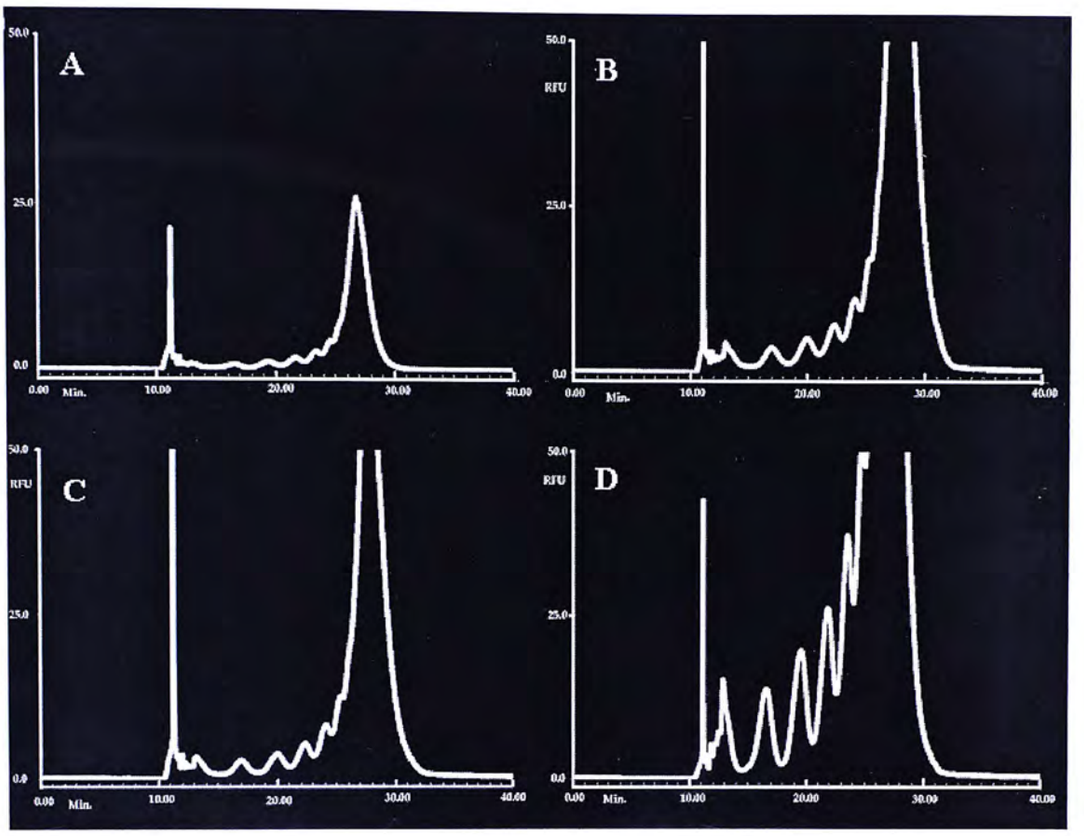


Figure 6.1. YC-1 caused dose-dependent enhancement of the apoptotic DNA fragmentation induced by serum deprivation in HRE-H9 cells. The data represent the CE-LIF electropherograms of the apoptotic DNA fragments isolated from the HRE-H9 cells in the different treatment conditions: (A) control cells with serum deprivation, (B) cells with both serum deprivation and treatment with 1 μM YC-1, (C) cells with both serum deprivation and treatment with 10 μM YC-1, and (D) cells with both serum deprivation and treatment with 100 μM YC-1. In all cases, the serum deprivation and exposure to YC-1 lasted for 24 h. Each electropherogram represents a sample from cells incubated in serum-free medium in a well of a 6-well culture plate. Samples in all electropherograms were from cells of the same passage in the same experiment. This electropherogram set is representative of 4 experiments.

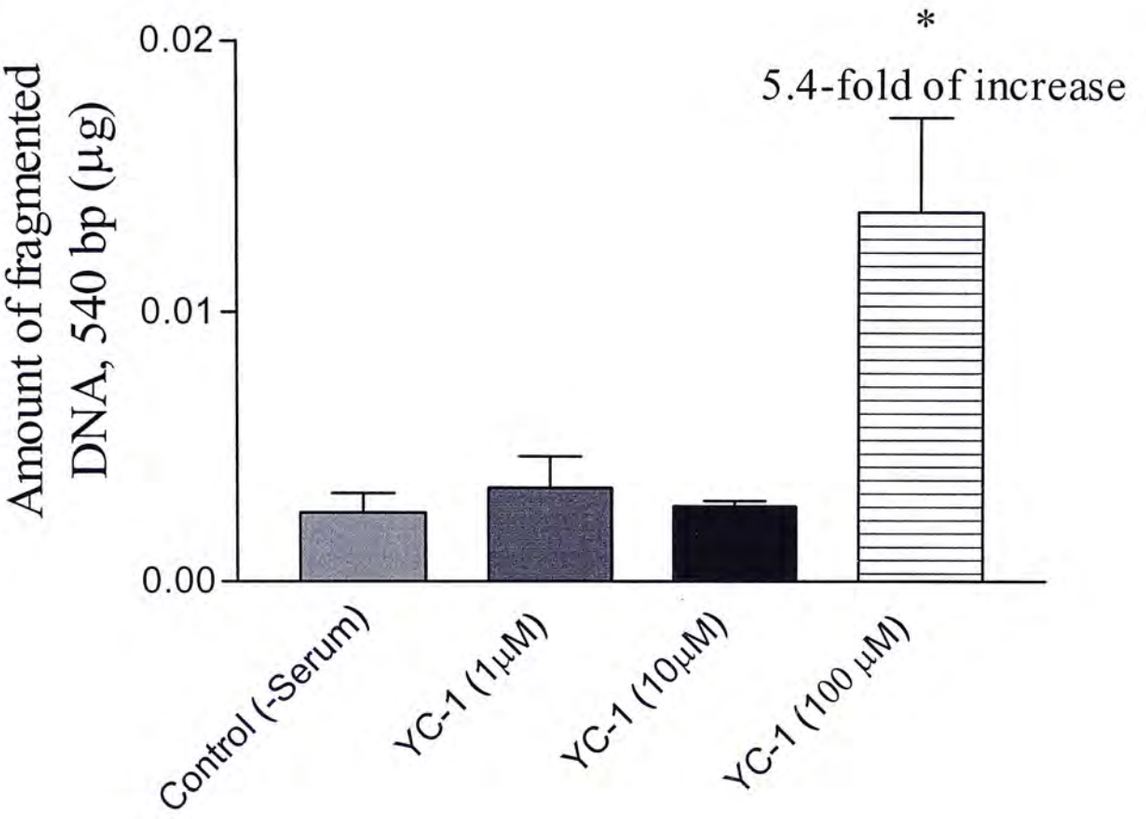


Figure 6.2. The combined data of 4 experiments showing the dose-dependency of the pro-apoptotic effects of YC-1 after 24 h of exposure. The amounts of the 540 bp DNA fragment were measured in samples from HRE-H9 cells in the different treatment conditions. The data represent the means ± SEM of n=4 samples per group. *P<0.05, comparing the treatment groups to the control. The number on the top of the bar indicates the fold increase in the amount of DNA fragmentation compared to control.

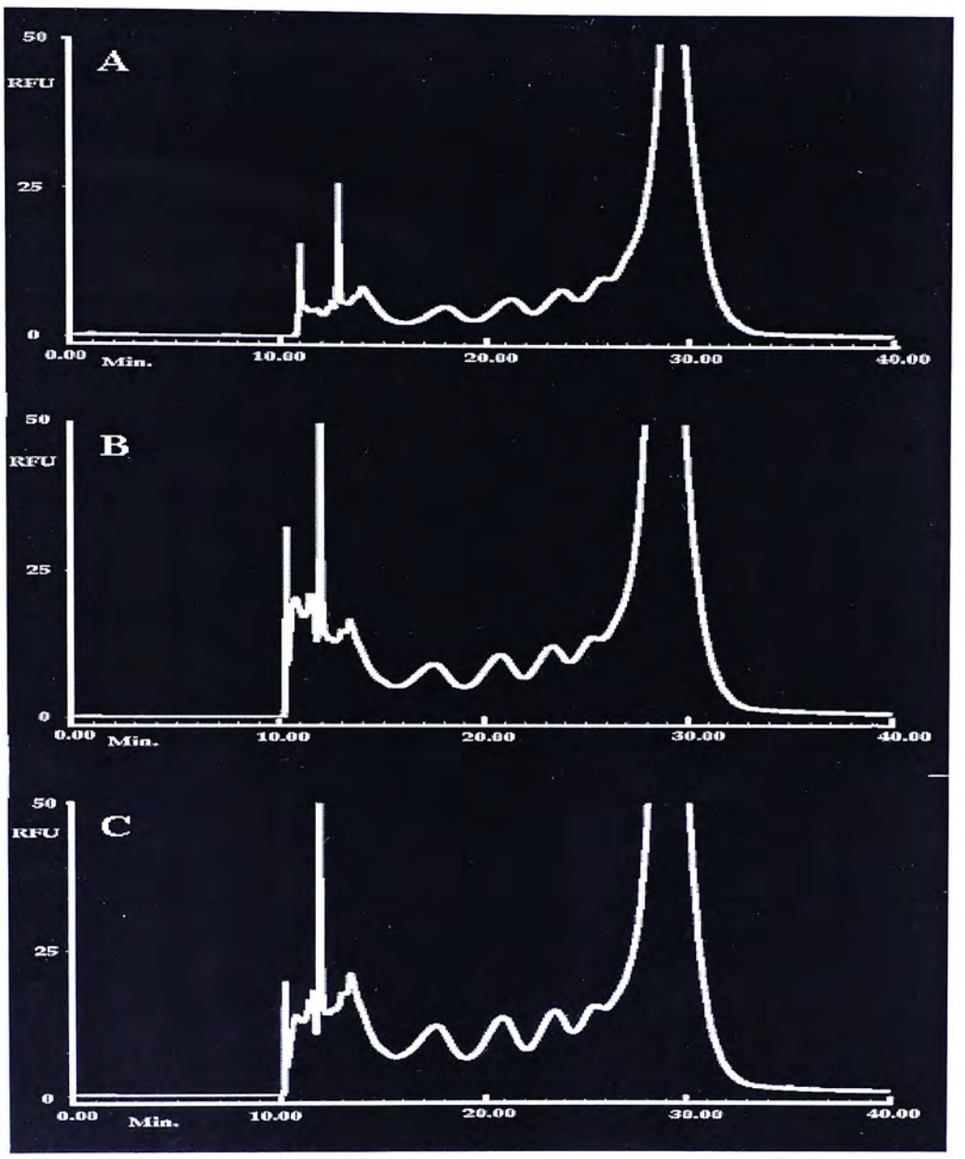


Figure 6.3. CE-LIF electropherograms of the apoptotic DNA fragments isolated from the HRE-H9 cells treated with 8-Br-cGMP. The conditions are: (A) control cells, (B) cells treated with 100 μM 8-Br-cGMP, and (C) cells treated with 1 mM 8-Br-cGMP. The exposure time in each case was 24 h. Each electropherogram represents a sample from cells incubated in a well of a 6-well culture plate containing medium supplemented with 4% fetal bovine serum. Samples in all electropherograms were from cells of the same passage in the same experiment. The electropherogram set is representative of 4 to 6 experiments.

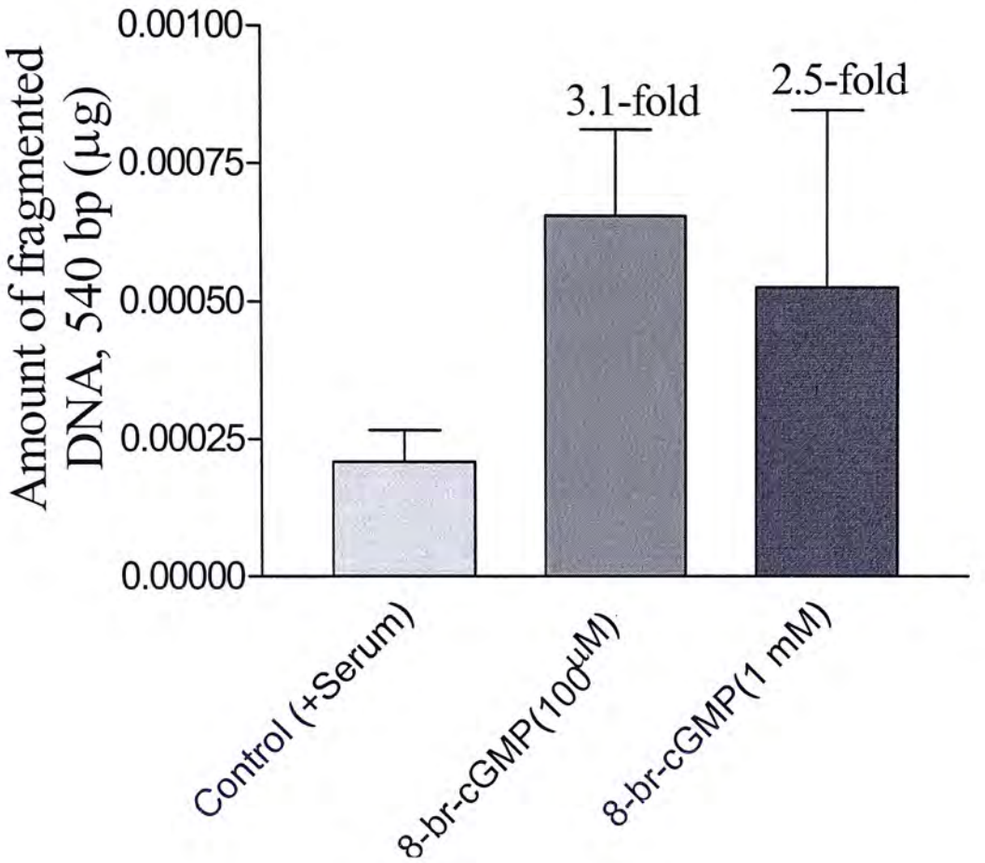


Figure 6.4. The combined data of 4-6 experiments showing that 8-Br-cGMP appears to increase apoptotic DNA fragmentation, but the effects were not statistically significant at the 5% level (i.e. $P>0.05$). The amounts of the 540 bp DNA fragment were measured in samples from HRE-H9 cells in the different treatment conditions. The data represent the means ± SEM of n=4-6 samples per group.

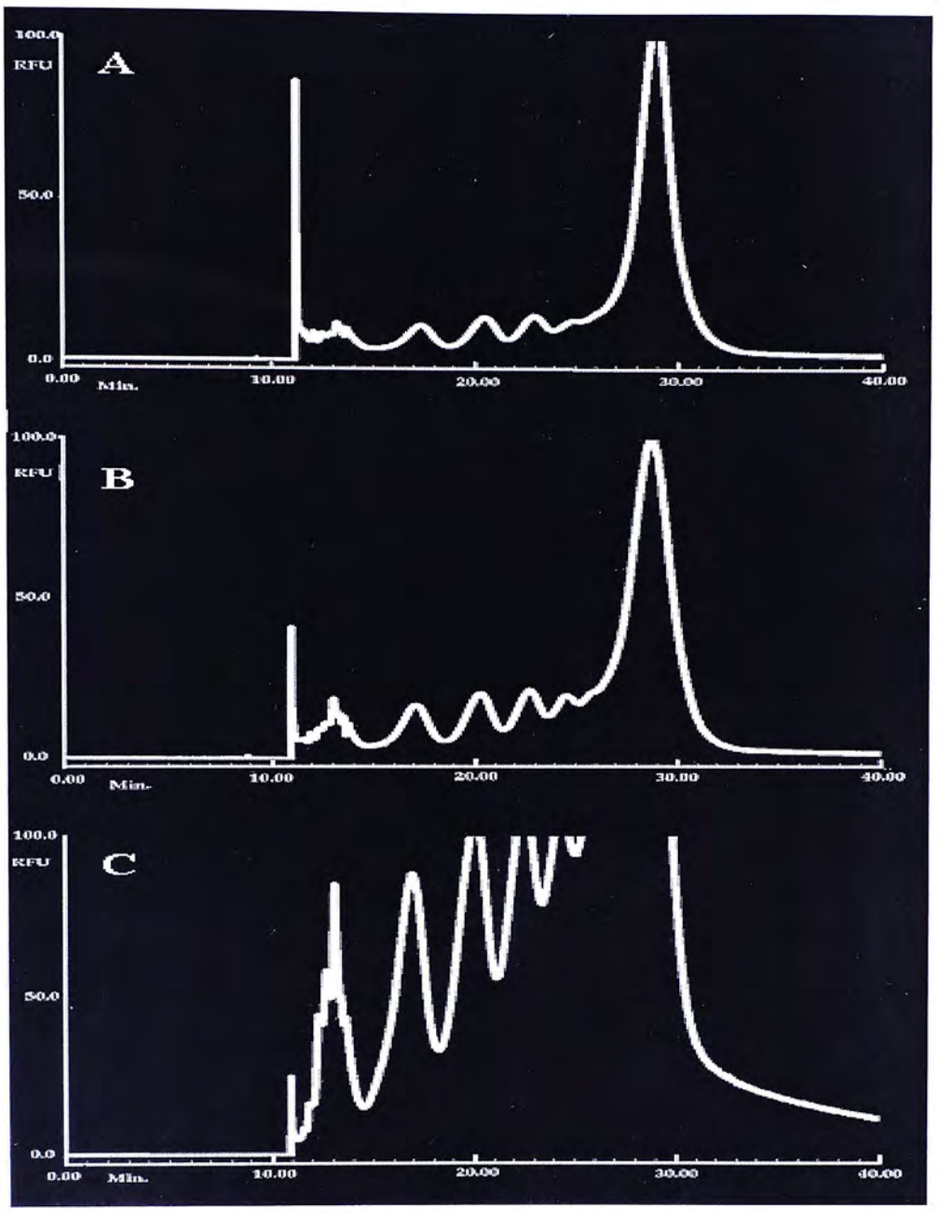


Figure 6.5. CE-LIF electropherograms of the apoptotic DNA fragments isolated from the HRE-H9 cells treated with 8-pCPT-cGMP in the presence of serum. The conditions were: (A) control cells, (B) cells treated with 100 μM 8-pCPT-cGMP, and (C) cells treated with 1 mM 8-pCPT-cGMP. The exposure time in each case was 24 h. Each electropherogram represents a sample from cells incubated in a well of a 6-well culture plate containing medium with 4 % fetal bovine serum. Samples in all electropherograms were from cells of the same passage in the same experiment. The electropherogram set is representative of 6 experiments.

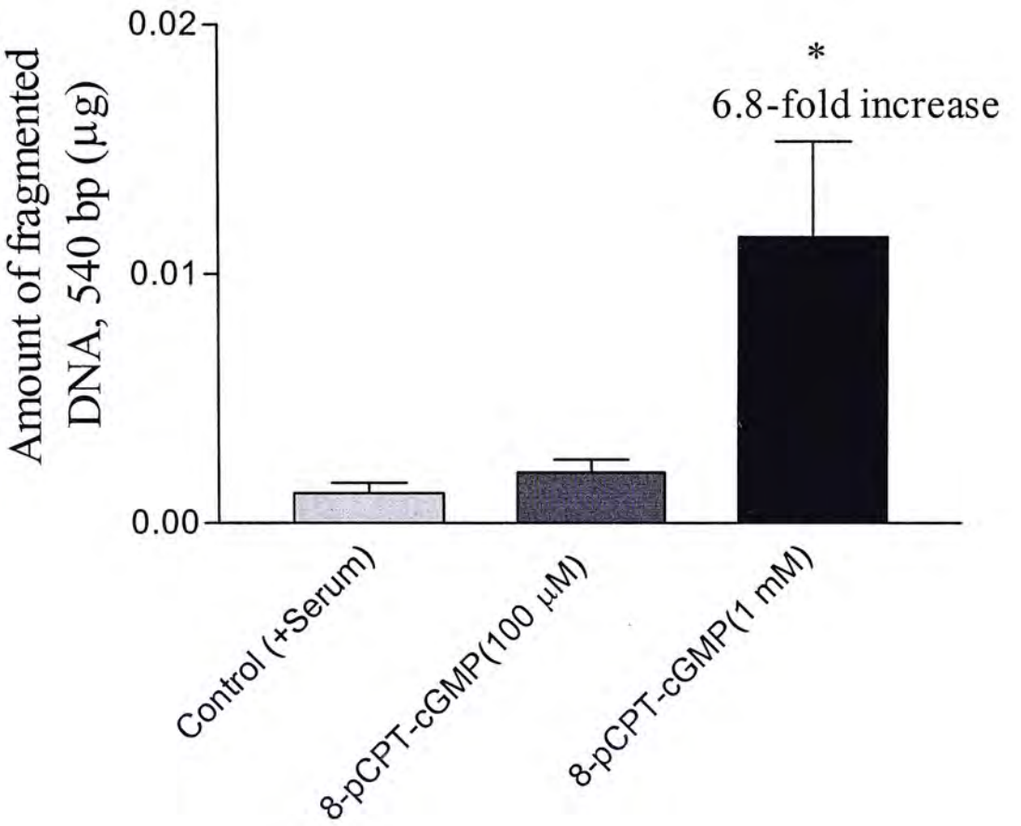


Figure 6.6. The combined data of 6 experiments showing the dose-dependency of the pro-apoptotic effect of 8-pCPT-cGMP (24 h). The amounts of the 540 bp DNA fragment were measured in samples from HRE-H9 cells in the different treatment conditions. The data represent the means ± SEM of n=6 samples per group. *P<0.05, comparing the treatment groups to the control. The number on the top of the bar indicates the fold-increase in the amount of DNA fragmentation compared to control.

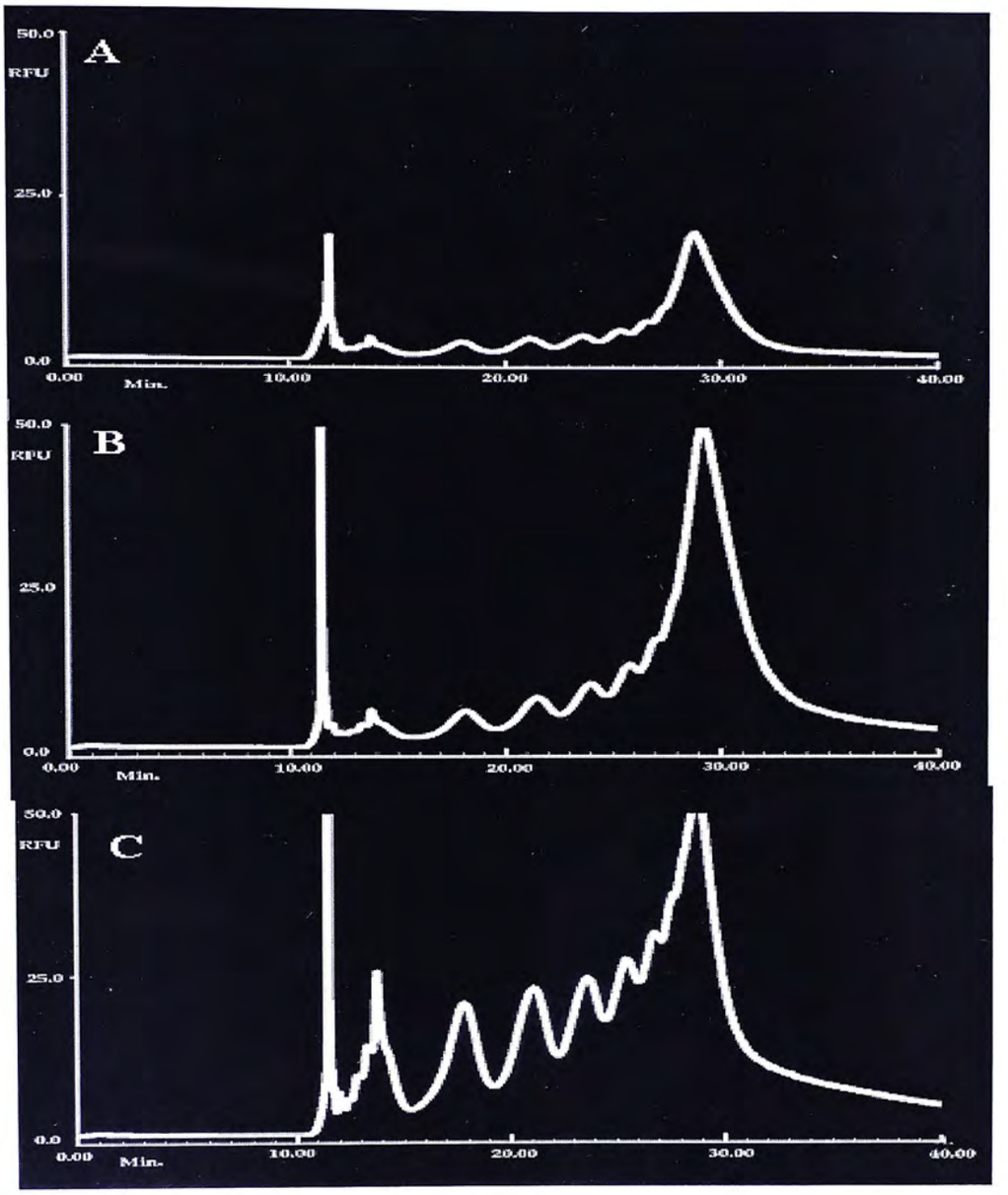


Figure 6.7. CE-LIF electropherograms of the apoptotic DNA fragments isolated from the HRE-H9 cells treated with 8-pCPT-cGMP under serum-free conditions. The conditions are: (A) control cells, (B) cells treated with 100 μM 8-pCPT-cGMP, and (C) cells treated with 1 mM 8-pCPT-cGMP. The exposure time in each case was 24 h. Each electropherogram represents a sample from cells incubated in a well of a 6- well culture plate containing medium without serum. Samples in all electropherograms were from cells of the same passage in the same experiment. The electropherogram set is representative of 5 experiments.

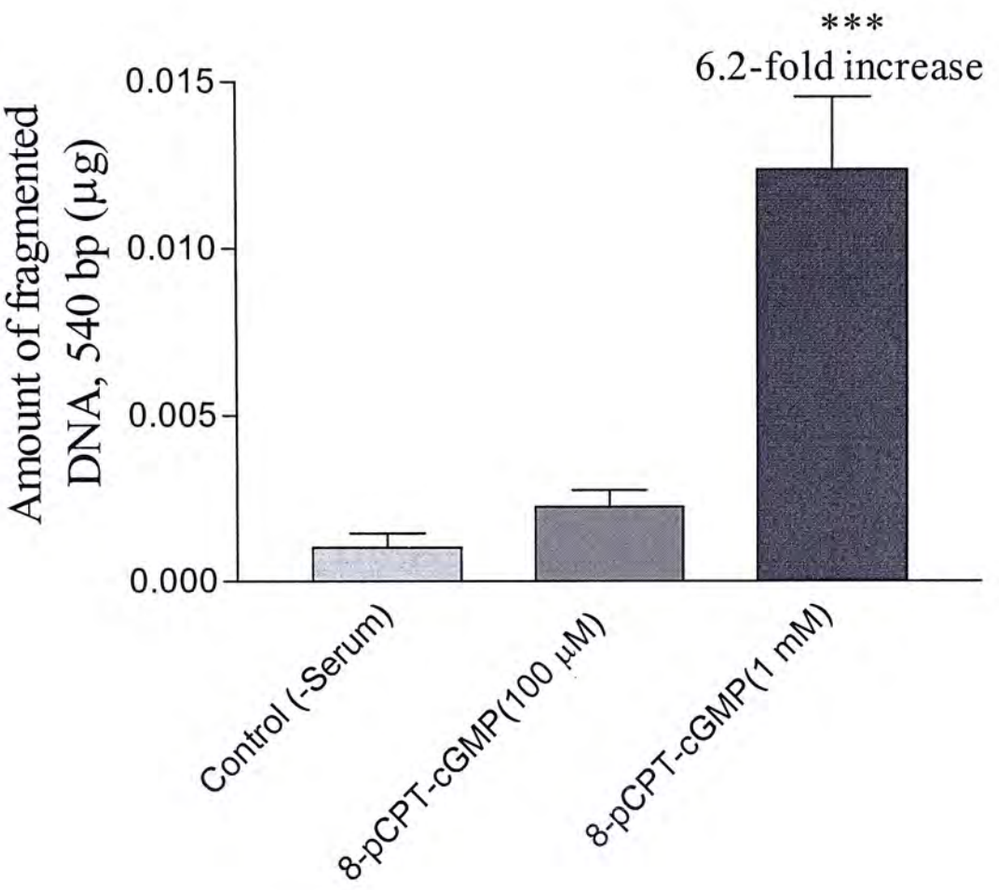


Figure 6.8. The combined data of 5 experiments showing the dose-dependency of the pro-apoptotic effects of 8-pCPT-cGMP (24 h) under serum-free conditions. The amounts of the 540 bp DNA fragment were measured in samples from HRE-H9 cells in the different treatment conditions. The data represent the means ± SEM of n=5 samples per group. *P<0.05, comparing the treatment groups to the control. The number on the top of the bar indicates the fold-increase in the amount of DNA fragmentation compared to control.

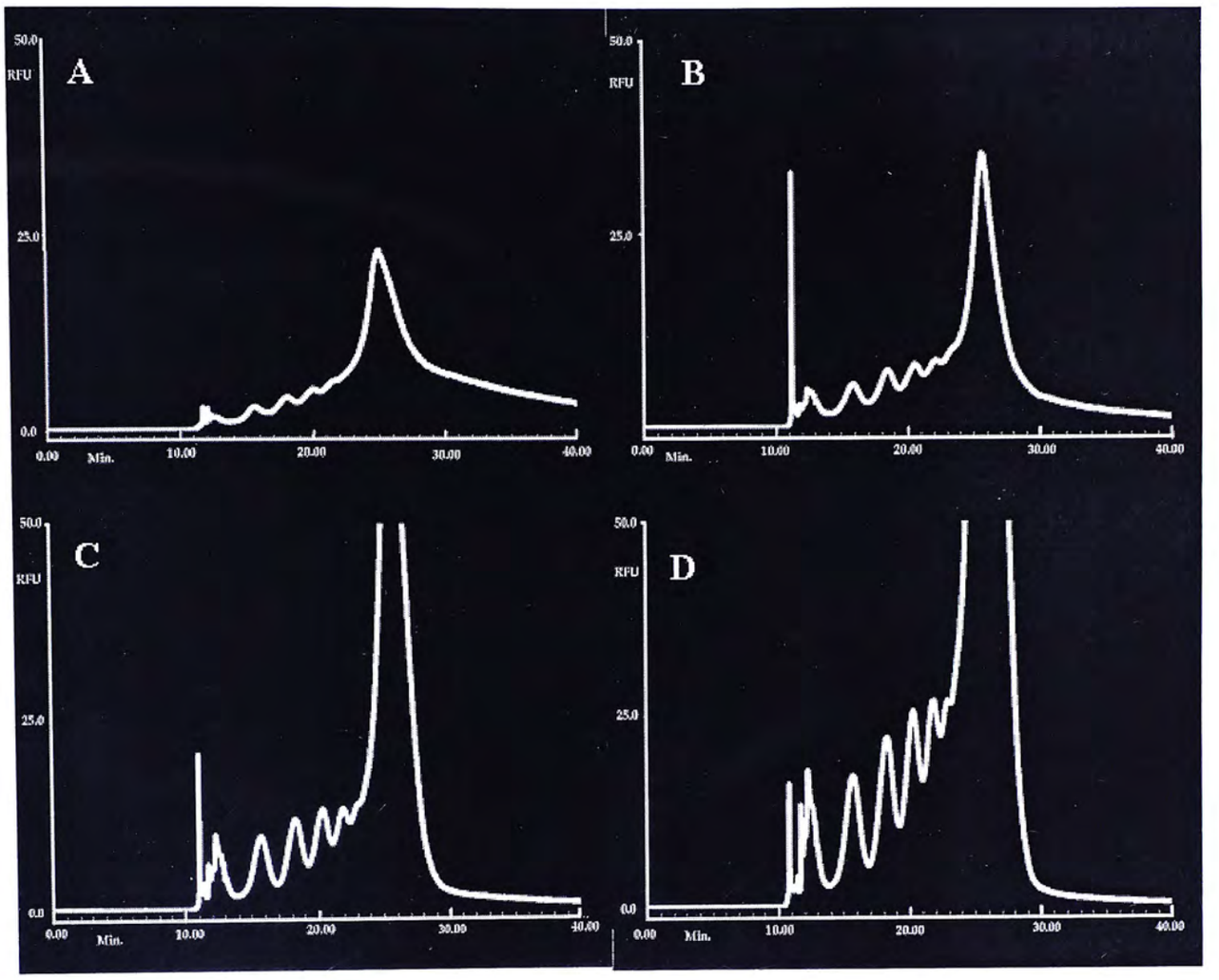


Figure 6.9. CE-LIF electropherograms of the apoptotic DNA fragments isolated from the HRE-H9 cells treated with Sp-8-Br-PET-cGMPS. The conditions are: (A) control cells, (B) cells treated with 1 μM Sp-8-Br-PET-cGMPS, (C) cells treated with 10 μM Sp-8-Br-PET-cGMPS, and (D) cells treated with 100 μM Sp-8-Br-PET-cGMPS. All cells were incubated in serum-free media. The exposure time in each case was 24 h. Each electropherogram represents a sample from cells in a well of a 6-well culture plate. Samples in all electropherograms were from cells of the same passage in the same experiment. The electropherogram set is representative of 5 experiments.

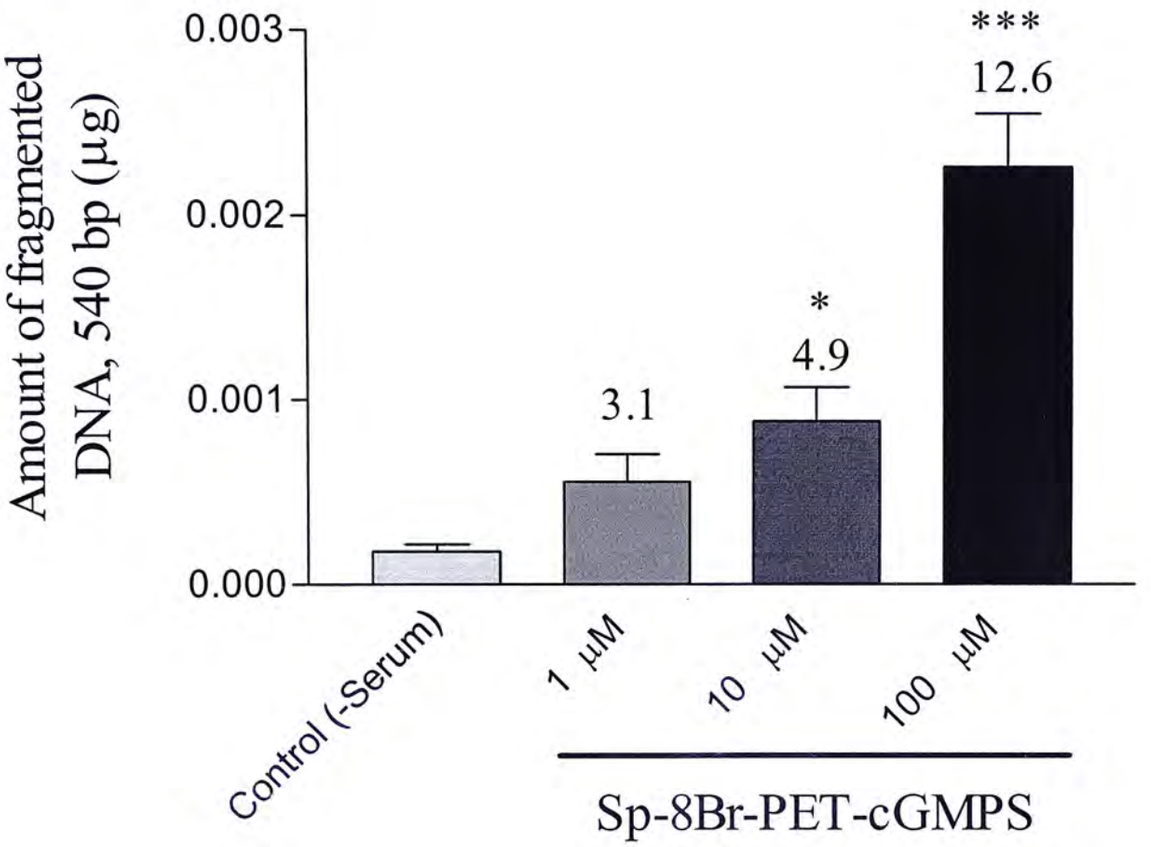


Figure 6.10. The combined data of 5 experiments showing the dose-dependency of the pro-apoptotic effects of Sp-8-Br-PET-cGMPS (24 h). The amounts of the 540 bp DNA fragment were measured in samples from HRE-H9 cells in the different treatment conditions. The data represent the means ± SEM of n=5 samples per group. *P<0.05, comparing the treatment group to the control. ***P<0.001, comparing the treatment group to the control. The number on the top of each bar indicates the fold-increase in the amount of DNA fragmentation compared to control.

Chapter 7: ODQ, an inhibitor of soluble guanylyl cyclase, down-regulates XIAP expression and induces apoptosis in human ovarian cancer cells

7.1 Abstract

Basal activity of soluble guanylate cyclase (sGC) and basal levels of cGMP appear to be necessary for the survival of a number of cells, including leukaemia cells, uterine epithelial cells as well as certain neural cells. X-linked inhibitor of apoptosis protein (XIAP), a member of the inhibitor of apoptosis protein (IAP) family, has been demonstrated to suppress apoptosis in human ovarian epithelial cancer cells. Until now, to our knowledge, there have been no reports concerning the effects of sGC on the regulation of apoptosis and the expression of XIAP in ovarian cancer cells. We hypothesized that basal sGC activity is anti-apoptotic in human ovarian cancer cells and that the anti-apoptotic function of the sGC/cGMP signaling pathway may involve the regulation of XIAP expression. In the present study, we determined the effects of the sGC inhibitor ODQ on XIAP level and apoptosis in a cisplatin-sensitive ovarian cancer cell line, A2780s cells. For comparison, we have also determined the effects of an agonistic monoclonal anti-Fas antibody (Fas mAb) on these parameters. To test whether the decrease in XIAP content by ODQ had contributed to the induction of apoptosis, we further determined if over-expression of XIAP could prevent apoptosis induced by ODQ. Exposure of A2780s cells to Fas mAb and ODQ for 24 h caused a concentration-dependent increase in

apoptosis, as measured by Hoechst staining. Whereas Fas mAb had no effect on XIAP levels, ODQ at 10, 50 and 100 μM significantly decreased XIAP content and increased the percentage of apoptotic cells by 2.3-, 6.0- and 7.8-fold, respectively. Over-expression of XIAP attenuated the ODQ-induced apoptosis in a concentration-dependent manner. These findings suggest that basal sGC activity and cGMP levels may be involved in preventing spontaneous apoptosis in ovarian cancer cells and that the down-regulation of XIAP may be an important event in the ODQ-induced apoptosis in A2780s ovarian cancer cells.

7.2 Introduction

Ovarian cancer is the most lethal gynaecological cancer in the Western world and ranks fifth among the most common female cancers (Wingo et al., 1995). Among all kinds of human ovarian malignancies, epithelial ovarian tumours, which originate from the surface epithelium of the ovary, account for over 85% (Neyns et al., 1996).

Tumour growth is now accepted to be the result of an imbalance between cell proliferation and apoptosis (Kerr et al., 1994; Levine et al., 1995; Sheets and Yeh, 1997; LaCass et al., 1998). Dysregulated apoptosis may contribute to the pathogenesis of a number of diseases, including cancer (Liebermann et al., 1995), autoimmune diseases (Reap et al., 1995), and many neurodegenerative disorders, including Alzheimer's disease

and Parkinson's disease (Estevez et al., 1998; Fiscus and Ming, 2000; Fiscus, 2002; Fiscus et al., 2002).

Recent research has demonstrated the presence of several potent endogenous suppressers of apoptosis, the inhibitor of apoptosis proteins (IAPs), in mammalian cells. IAPs were first identified in baculoviruses, where they keep the host cells alive while the viruses replicate (Birnbaum et al., 1994; Crook et al., 1993). The IAP family currently consist of six members: neuronal apoptosis inhibitory protein [NAIP; Roy et al., 1995], X-linked inhibitor of apoptosis protein (XIAP; Liston et al., 1996; Uren et al., 1996; Duckett et al., 1996), human inhibitor of apoptosis protein-1 (HIAP-1) (Liston et al., 1996; Uren et al., 1996; Duckett et al., 1996), human inhibitor of apoptosis protein-2 (HIAP-2; Liston et al., 1996; Uren et al., 1996; Rothe et al., 1995), survivin (Ambrosini et al., 1997) and Livin (Kasof and Gomes, 2001). With the exception of NAIP and survivin, which only have the N-terminal repeats named baculovirus IAP repeats (BIRs; required for biologic function), all the other mammalian IAPs identified to date possess both N-terminal BIRs and a C-terminal RING-zinc finger domain, which is required for protein-protein interaction.

The role of XIAP in the regulation of apoptosis in human ovarian cancer has been examined recently. XIAP is localized in human ovarian carcinomas, in which there are higher levels of XIAP in proliferative epithelial cancer cells compared to apoptotic epithelial cancer cells (Li et al., 2000; Li et al., 2001a). The pro-apoptotic agent cisplatin, a

chemotherapeutic agent commonly used in the treatment of ovarian cancer, has been shown to decrease XIAP content and induce apoptosis (Li et al., 2000; Li et al., 2001a). XIAP is known to suppress apoptosis via caspase -3 and -7 inhibition (Deverasaux et al., 1997; Takahashi et al., 1998) as well as interfering with the Bax/cytochrome c pathway by inhibiting caspase-9 (Deveraus et al., 1998, 1999). Over-expression of IAPs has been shown to suppress apoptosis in mammalian cells. For example, over-expression of NAIP, XIAP, HIAP-1 or HIAP-2 protects Chinese hamster ovary (CHO) and RAT-1 cells from apoptosis triggered by menadione (Liston et al., 1996) and over-expression of XIAP or HIAP-2 protects HeLa cells from apoptosis induced by transient transfection of pro-interleukin-1β-converting enzyme (Uren et al., 1996). Furthermore, the laboratory of Professor Benjamin K. Tsang of the University of Ottawa, collaborator on this part of the thesis project, has recently shown that over-expression of XIAP in human ovarian epithelial cancer cells markedly attenuates cisplatin-induced apopotosis (Asselin et al., 2001; Li et al., 2001a). Although Hiap-2 is present in A2780s cell lines, it content in the cell lines was not significantly affected by cisplatin and decreased to a lesser extent than Xiap in OV2008, suggesting a less important role for Hiap-2 in ovarian tumor biology.

Soluble guanylate cyclase (sGC), the enzyme responsible for the synthesis of most of the basal cGMP in mammalian cells (Waldman and Murad, 1987; Garthwaite et a., 1995; Fiscus, 2002), has been demonstrated to be necessary for the survival of L1210 leukemia cells under normal growing conditions as well as for protection against various apoptotic stimuli (Flamigni et al., 2001). Our previous studies have demonstrated that the basal

activities of sGC and a downstream target protein in the cGMP signaling pathway, protein kinase G (PKG), may be important for the survival of uterine epithelial cells (Chan and Fiscus, 2002) and several neural cell lines (Yuen and Fiscus, 2001; Fiscus, 2002). Specifically, basal activities of sGC and PKG appear to protect these cells against spontaneous apoptosis. We have hypothesized that basal sGC activity and cGMP levels are anti-apoptotic in human ovarian cancer cells, and that this effect involves in part the regulation of XIAP expression. Until now, to our knowledge, there have been no reports concerning the effects of sGC on the regulation of XIAP expression and apoptosis in ovarian cancer cells.

In the present study, we have determined the effects of the sGC inhibitor ODQ on XIAP content and apoptosis in the ovarian epithelial cancer cell line A20780s. For comparison, we have also determined if an agonistic monoclonal anti-Fas antibody alters these parameters. Previous studies in the laboratory of Professor Benjamin K. Tsang had shown that the anti-Fas antibody decreased XIAP level and induced apoptosis in another ovarian cancer cell line, OV2008 (unpublished data). In the present study, we found that ODQ and anti-Fas antibody both significantly increase the levels of apoptosis in A2780s cells. However, only ODQ significantly decreased XIAP levels in these cells. Over-expression of XIAP in A2780s cells completely prevented apoptosis induced by ODQ. These findings support the concept that ODQ induces apoptosis through down-regulation of XIAP content in ovarian cancer cells.

7.3 Results

Induction of apoptosis, but no change in XIAP levels, by an agonistic Fas mAb in A2780s cells

A2780s cells cultured in the absence of an exogenous agonist exhibited normal cellular morphology, as evident by their adherence to the growth surface and flat, cobblestone-like appearance. Addition of an agonistic monoclonal anti-Fas antibody (Fas mAb) induced apoptotic features, including cell rounding and shrinkage as well as nuclear condensation and fragmentation (Figure 7.1A). The apoptosis induced in the A2780s cells was significant at all three concentrations (0.5, 1.0 and 2.0 μg/ml) of Fas mAb tested (Figure 7.1B). Fas mAb, at the same concentrations, had no detectable effects on the levels of XIAP in the A2780s cells (Figure 7.2)

Induction of apoptosis by the soluble guanylyl cyclase (sGC) inhibitor ODQ in A2780s cells

Previously, we had shown that ODQ induces apoptosis in an immortalized uterine epithelial cell line, the HRE-H9 cells (Chan and Fiscus, 2002), and two established neural cell lines (N1E-115 and NG108-15 cells) (Yuen and Fiscus, 2002; Fiscus, 2002), suggesting that basal levels of sGC activity are important for preventing spontaneous

induction of apoptosis in these cells. To examine whether basal levels of sGC modulate the apoptosis of ovarian cancer cells, the A2780s cells were incubated in the presence or absence of ODQ, a potent and selective inhibitor of sGC (Garthwaite et al., 1995; Moro et al., 1996; Mulsch et al., 1997; Olesen et al., 1998). Our previous studies had shown that ODQ at 40 μM (24 h exposure) completely inhibited sGC activity, lowered basal levels of cGMP to one-fifth of normal levels and induced apoptosis in two neural cell lines, NG108-15 cells (Yuen and Fiscus, 2001; Fiscus et al., 2002) and NIE-115 cells (Fiscus et al., 2002). In the present study, we found that exposure of A2780s cells to ODQ (1-100 μM) for 24 h caused a concentration-dependent increase in apoptosis, as measured by Hoechst nuclear staining (Figure 7.3). While ODQ at 1 μM had no significant effect on A2780s cell apoptosis when compared with the controls, it significantly increased nuclear chromatin condensation and fragmentation in the A2780s cells at 10, 50 and 100 μM. At these concentrations, ODQ caused 2.3-, 6.0- and 7.8-fold increases in the percentage of apoptotic cells (Figure 7.4) suggesting that adequate basal sGC activity and cGMP levels may be important in preventing spontaneous apoptosis in the ovarian cancer cells.

ODQ down-regulates XIAP expression in A2780s cells

To determine whether XIAP down-regulation could be associated with the induction of apoptosis by ODQ, changes in XIAP content in A2780s cells incubated with ODQ (1-100 μM) for 24 h were assessed by Western blot analysis. ODQ induced morphological

changes indicative of apoptosis in some of the A2780s cells as shown in Figure 7.3. The increase in the incidence of apoptosis was accompanied by significant concentration-dependent decreases in XIAP content and to a smaller extent in β-tubulin content (Figure 7.5A). GAPDH content however, was not significantly affected by the ODQ treatment, and was thus used to normalize the XIAP levels (Figure 7.5A). Statistical analysis of the quantified XIAP protein level in relation to those of GAPDH revealed that ODQ significantly reduced XIAP levels in the A2780s cells in a concentration-dependent manner ($P<0.001$) and that at 10 μM of ODQ, XIAP content was approximately 50% of that in the control cells (Figure 7.5B). Thus, at concentrations that induced apoptosis, ODQ significantly decreased the content of the anti-apoptotic protein XIAP, suggesting that a down-regulation of XIAP level may be a contributing event in ODQ-induced apoptosis in these ovarian cancer cells.

XIAP over-expression prevents ODQ-induced apoptosis in A2780s cells

To determine whether a decrease in XIAP content was indeed an important determinant in ODQ-induced apoptosis in A2780s cells, the influence of XIAP over-expression on the ability of ODQ to induce apoptosis in A2780s cells was determined after infection of the ovarian cancer cells with adenoviral full-length XIAP sense cDNA. XIAP over-expression caused a dose-dependent protection of the A2780s cells against the ODQ-induced apoptosis (Figure 7.6). At MOI = 10, XIAP over-expression completely prevented apoptosis induced by ODQ. These data confirm that the XIAP down-regulation may be an

important element in the ODQ-induced apoptosis in A2780s cells. Figure 7.7 shows that ODQ reduced XIAP protein content in A2780s cells infected 48 h earlier with adenoviral Lac Z (control vector) but not with adenoviral XIAP sense cDNA. Adenoviral XIAP sense infection elevated the XIAP level significantly above the control levels ($P<0.001$; Figure 7.7B)

7.4 Discussion

The soluble form of guanylyl cyclase (sGC) is a heme-containing enzyme that is activated by the binding of nitric oxide (NO) to the heme moiety (Waldman and Murad, 1987). Beauvais and coworkers have shown that activation of sGC by NO donors and the subsequent elevation of cGMP levels result in an inhibition of the apoptosis induced by cytokine deprivation in eosinophils (Beauvais et al., 1995). Since this early report, several other types of cells, most notably neural cells, have been shown to be protected against induction of apoptosis when cGMP levels are elevated by the activation of either sGC or particulate guanylyl cyclase (Kim et al., 1999; Fiscus et al., 2001b; Fiscus, 2002; Fiscus et al., 2002).

Even in the absence of NO, sGC has basal activity, contributing to most of the basal cGMP levels found in mammalian cells (Waldman and Murad, 1987; Garthwaite et al. 1995; Fiscus, 2002). Inhibition of this basal sGC activity, using specific sGC inhibitors

like ODQ, can result in the lowing of the basal levels of cGMP (Garthwaite et al., 1995; Flamigni et al., 2001; Fiscus, 2002). Several recent studies have suggested that basal sGC activity and basal cGMP levels may be sufficient to activate, at least partially, the cGMP-mediated anti-apoptotic/pro-survival pathway in certain mammalian cells, thus protecting these cells against spontaneous apoptosis. For example, inhibition of sGC by ODQ was found to induce marked increases in caspase activity, which was associated with a loss of cell viability and a reduction in cGMP content in L1210 leukemia cells (Flamigni et al., 2001). Furthermore, ODQ provoked the processing of caspase-3 and -9, release of cytochrome c, reduction of Bcl-2 content and dephosphorylation of Bad at Ser 112 in the L1210 leukaemia cells. Thus, ODQ and the inhibition of sGC appear to alter a number of apoptosis-regulating pathways, ultimately resulting in the induction of apoptosis in the leukaemia cells. These data suggest that the basal activity of sGC is essential in preventing a spontaneous activation of pro-apoptotic pathways, at least in leukemia cells. Our recent studies have demonstrated that the basal activities of sGC and a downstream target protein in the cGMP signaling pathway, protein kinase G (PKG), may be important for the survival of two other types of cells, an immortalized uterine epithelial cell line (Chan and Fiscus, 2002) and two established neural cell lines (Yuen and Fiscus, 2001; Fiscus, 2002). Specifically, we have found that basal activities of sGC and PKG are necessary to protect these cells against a spontaneous induction of apoptosis under normal growing conditions.

The laboratory of Professor Benjamin K. Tsang has previously shown that addition of cisplatin to human ovarian surface epithelial cancer cell cultures decreases XIAP protein content and induces apoptosis in cisplatin-sensitive ovarian cancer cells (Li et al., 2000a; Li et al., 2001a). Over-expression of XIAP in these cells by adenoviral sense XIAP cDNA infection effectively attenuates cisplatin-induced apoptosis (Li et al., 2001a). In the present study, we hypothesized that basal sGC activity and basal levels of cGMP may cause anti-apoptotic effects in human ovarian cancer cells and that this effect may involve the regulation of XIAP expression. Until now, to our knowledge, there have been no reports concerning the effects of sGC on apoptosis as well as on the regulation of the expression of XIAP in ovarian cancer cells. In the present study, we determined the effects of the sGC inhibitor ODQ on the regulation of XIAP content and apoptosis in the cisplatin-sensitive ovarian cancer cell line A2780s. The present study also tested the effects of an agonistic Fas monoclonal antibody (Fas mAb) on XIAP level and apoptosis in the cells. Previously, we had found that Fas mAb induces apoptosis (Schneiderman et al., 1999) and down-regulates XIAP expression (unpublished data) in another cisplatin-sensitive ovarian cancer cell line.

In the present study, an agonistic Fas mAb significantly induced apoptosis in A2780s cells in a concentration-dependent manner, as evident by the presence of cellular condensation, nuclear fragmentation and apoptotic bodies, but did not down-regulate the XIAP level. In contrast, ODQ concentration-dependently decreased the XIAP content and

induced apoptosis in A2780s cells. The findings suggest that the basal sGC activity is essential to protect the A2780s cells against a spontaneous apoptosis, as previously shown in uterine epithelial cells (Chan and Fiscus, 2002) and neural cells (Yuen and Fiscus, 2001; Fiscus, 2002). Furthermore, because the increase in apoptosis was accompanied by a decrease in XIAP content, we suspected that the ODQ-induced apoptosis in the A2780s cells had resulted, at least in part, from a decrease in XIAP content and thus the subsequent loss of an anti-apoptotic mechanism within the cell. The precise mechanism of the ODQ-induced decrease in XIAP content in A2780s cells is currently not known. In addition to suppressed gene expression, it is possible that the decreased XIAP content could be a consequence of increased XIAP ubiquitination / proteosome degradation (Yang et al 2000) or caspase-3-mediated processing (Deveraux et al 1999). Nonetheless, the present data suggest that basal sGC activity and basal levels of cGMP may be necessary for the maintenance of a sufficiently high level of XIAP in the cisplatin-sensitive human ovarian cancer cells to ensure cell survival and growth under normal conditions.

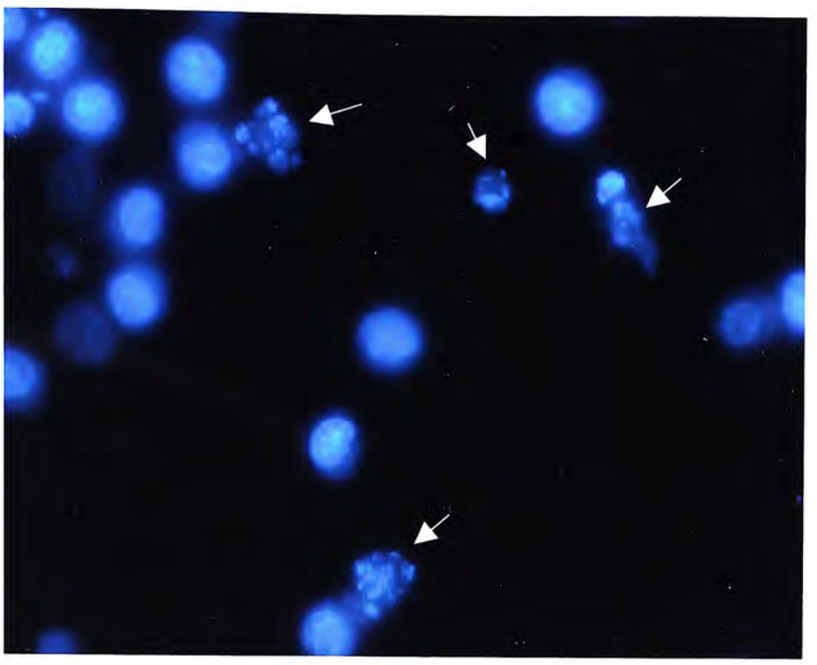
To further confirm the contribution of XIAP down-regulation in the ODQ-induced apoptosis, we determined if XIAP over-expression could prevent the apoptosis induced by ODQ. XIAP over-expression by adenoviral infection with sense XIAP cDNA resulted in dose-dependent protection, and at the highest level of over-expression complete protection, of the A2780s cells from the ODQ-induced apoptosis. These data thus add further support

for the concept that ODQ, via inhibition of sGC and lowering of basal cGMP levels, induces apoptosis, at least in part, by down-regulating XIAP level in ovarian cancer cells.

The mechanisms by which XIAP exert its anti-apoptotic effects are still not fully understood. The IAPs have been shown to directly inhibit certain cell-death proteases, such as caspase-3, caspase-7 (Daveraux et al., 1997; Roy et al., 1997; Takahashi et al., 1998) and caspase-9 (Deveraux et al., 1999). Furthermore, XIAP expression levels appear to regulate the activation of caspases-3 and caspases-9. For example, the laboratory of Professor Benjamin K. Tsang has shown that XIAP down-regulation following antisense infection in A2780s cells causes caspases-3 activation (Sasaki et al., 2000). Over-expression of XIAP blocks the cisplatin-induced cleavage of caspases-9 and caspases-3 in A2780s cells (Asselin et al., 2001). Thus, the induction of apoptosis in A2780s cells by ODQ in the present study may have resulted from the lowering of XIAP content, which would be expected to activate caspases as well as cause a loss of endogenous inhibition of caspase activities. If a similar mechanism is present in leukaemia cells, this proposed action of ODQ on the down-regulation of XIAP expression may have also contributed to the ODQ-induced activation of caspases and onset of apoptosis observed in L1210 leukaemia cells (Flamigni et al., 2001). Additional studies will be needed to establish whether caspase-3 or other cell-death proteases are involved in the ODQ-induced apoptosis in ovarian cancer cells.

In summary, we have shown that the sGC inhibitor ODQ down-regulates XIAP content and induces apoptosis in the human ovarian cancer cell line A2780s. Preventing the decrease in XIAP content by over-expressing this protein completely protected the cells against ODQ-induced apoptosis. Our findings suggest that basal sGC activity and cGMP level may be necessary for the maintenance of high XIAP level for prevention of spontaneous apoptosis in these ovarian cancer cells under normal culture conditions. These findings provide a new concept for the regulation of apoptosis in ovarian cancer cells and of ovarian tumor growth, showing a potential link between the anti-apoptotic effects of the sGC/cGMP signaling pathway and the cell survival role of XIAP.

A



B

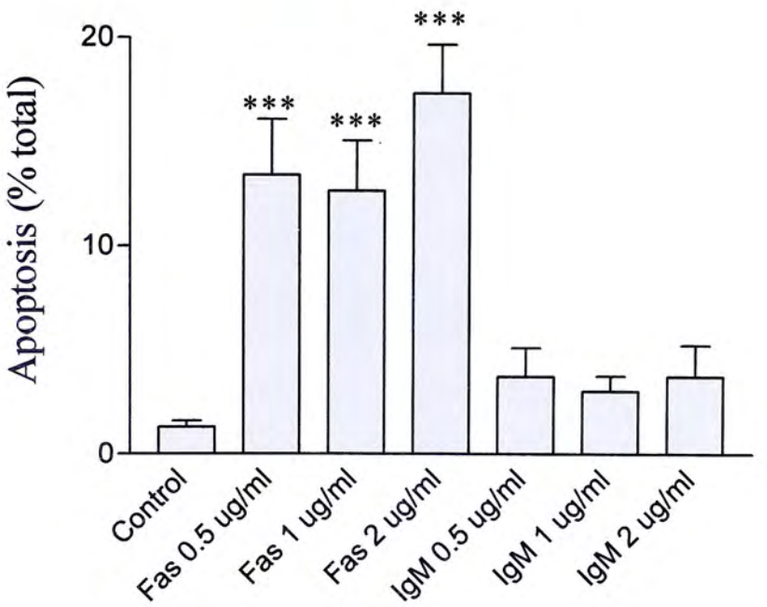


Figure 7.1. (A) Representative photomicrograph showing apoptotic nuclear morphology (Hoechst staining) in the A2780s ovarian epithelial cancer cells after treatment for 24 h with an agonistic Fas mAb (2 μg/ml). The white arrows indicate nuclear fragmentation. Magnification: 400X. (B) Effect of Fas mAb, at selected concentrations, on the levels of apoptosis in A2780s cells. The cells were plated at a density of 5 x 10^4 cells/cm^2 in 6-well plates and incubated for 24 h in DMEM-F12-10% FBS followed by incubation for an additional 24 h in serum-free DMEM-F12 with different concentrations of Fas mAb (0, 0.5, 1 and 2 μg/ml). The data represent the means ± SEM of n=3 samples per group. ***P<0.001, comparing the treatment groups to the control.

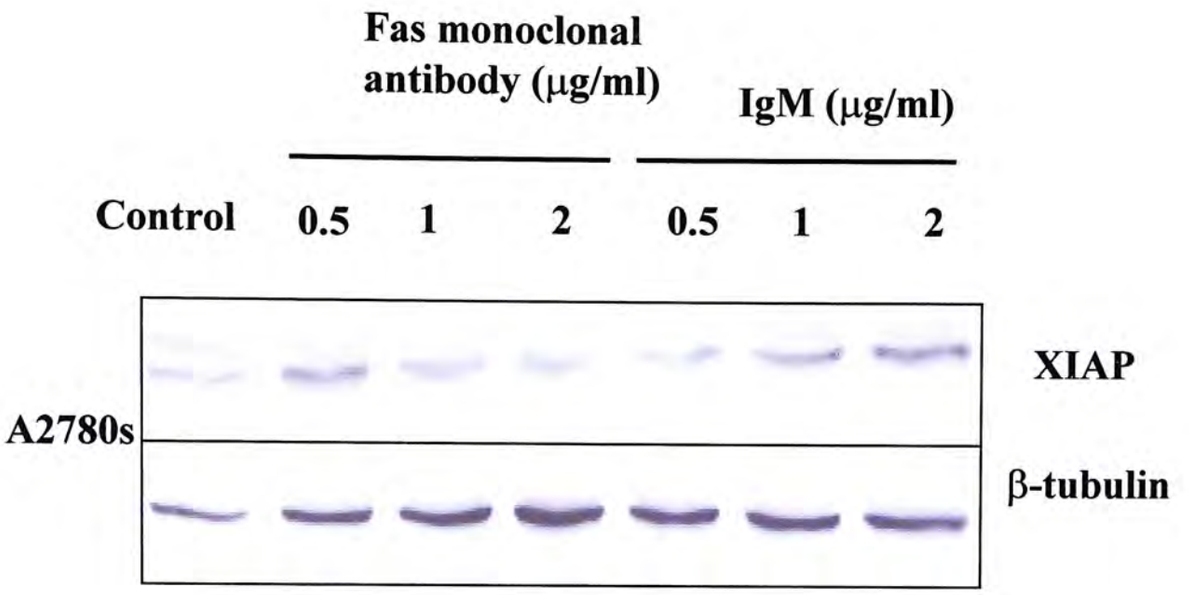


Figure 7.2. Effect of Fas mAb, at the same concentrations that had resulted in increased levels of apoptosis, on XIAP protein content in A2780s cells, assessed by Western blot analysis. The Fas mAb has no detectible effect on XIAP protein content. The data are representative of three independent experiments.

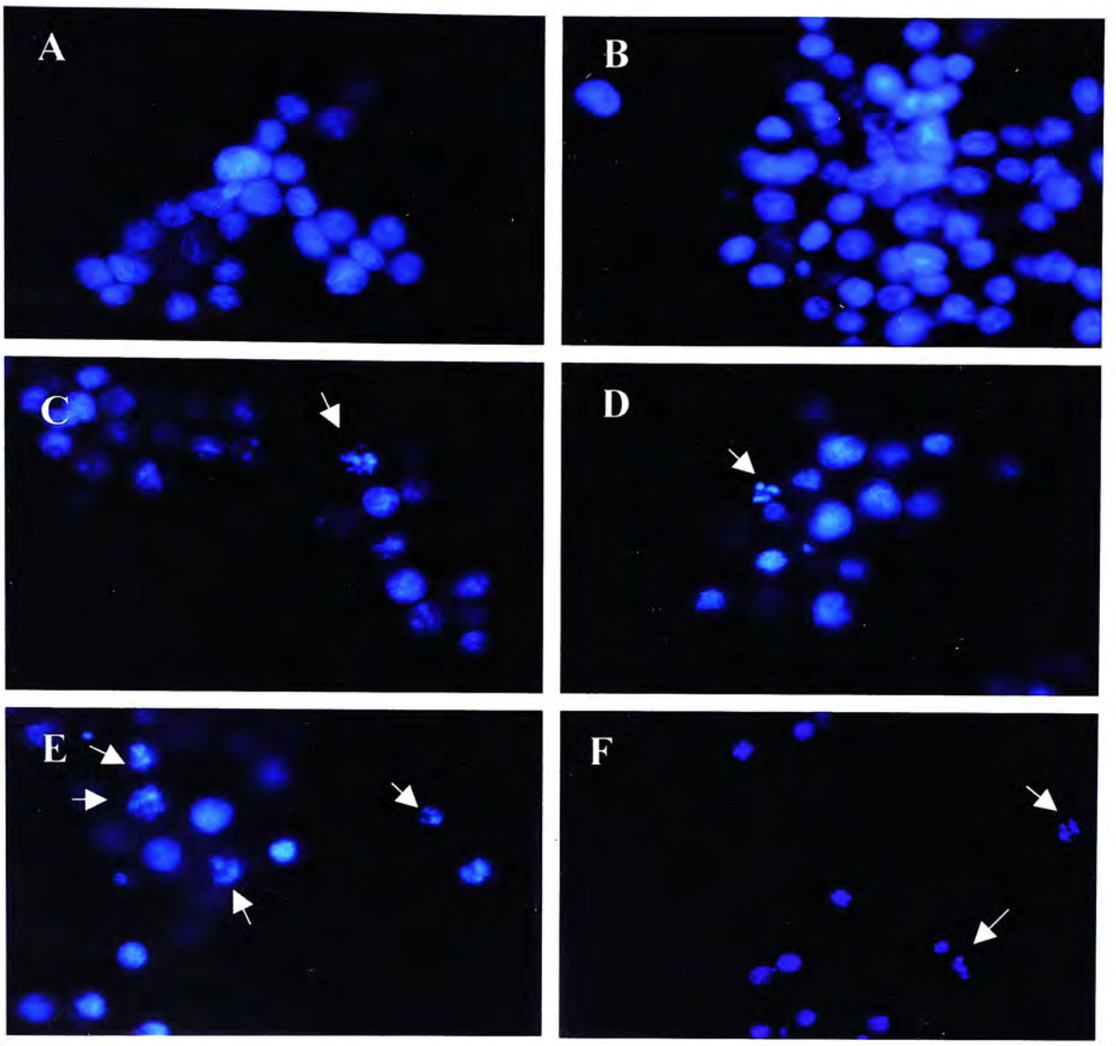


Figure 7.3. Representative photomicrographs showing apoptotic nuclear morphology (Hoechst staining) in A2780s cells after treatment for 24 h with the sGC inhibitor ODQ. Cells were plated at a density of 5 x 10^4 cells/cm^2 in 6-well plates, incubated for 24 h in DMEM-F12-10% FBS and further incubated for an additional 24 h in serum-free DMEM-F12 with ODQ (0, 1, 10, 50 and 100 μM). (A) Cells treated with 0 μM ODQ (Control) (400X). (B) Cells treated with 1 μM ODQ (400X). (C) Cells treated with 10 μM ODQ (400X). (D) Cells treated with 50 μM ODQ (400X). (E) Cells treated with 100 μM ODQ (400X). (F) Cells treated with 100 μM ODQ (200X). White arrows indicate nuclear fragmentation.

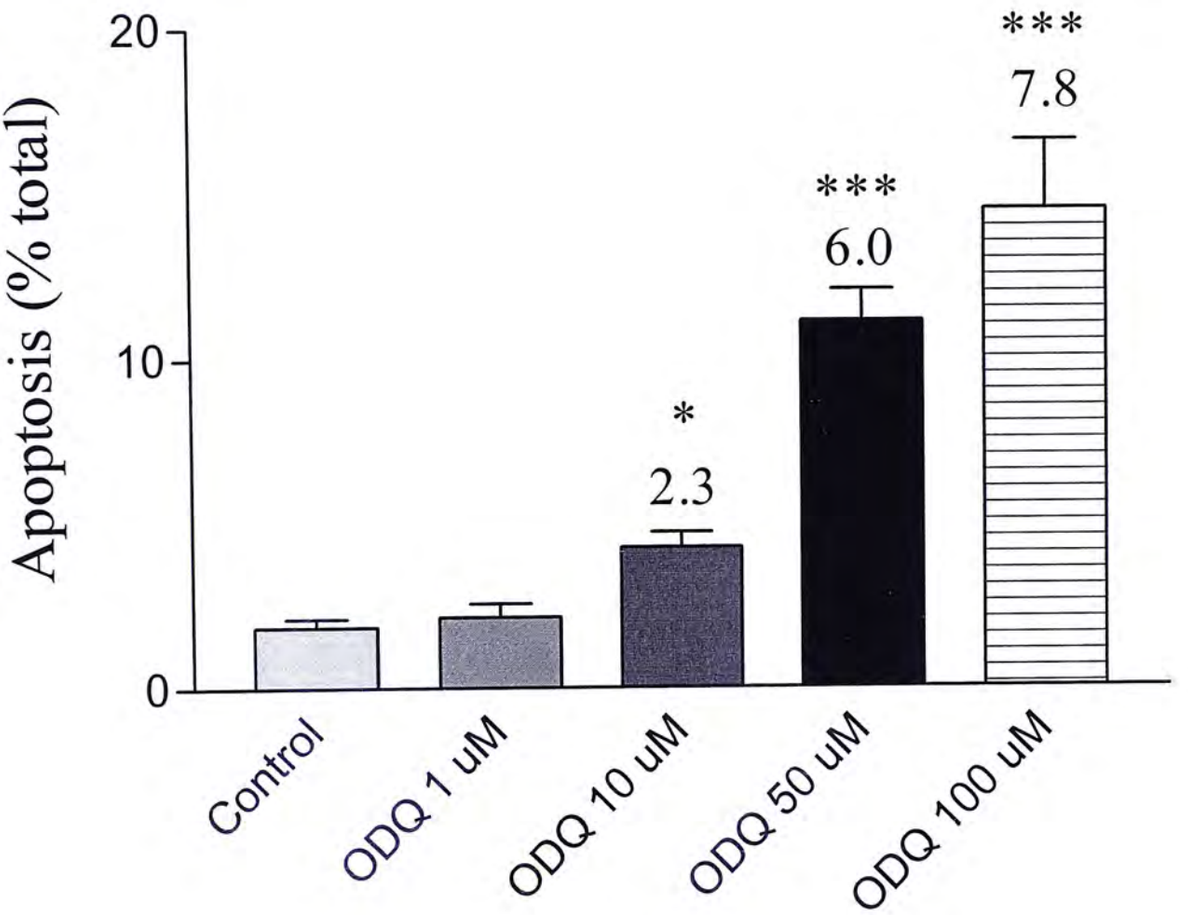


Figure 7.4. Effect of ODQ at different concentrations on apoptosis in A2780s cells. Cells were treated with ODQ and collected for Hoechst staining, as in Figure 7.3. The percentage of cells with apoptotic nuclear morphology are indicated (combined data of five experiments). The data represent the means ± SEM of n=3-5 samples per group. *P<0.05 and ***P < 0.001, comparing the treatment groups to the control.

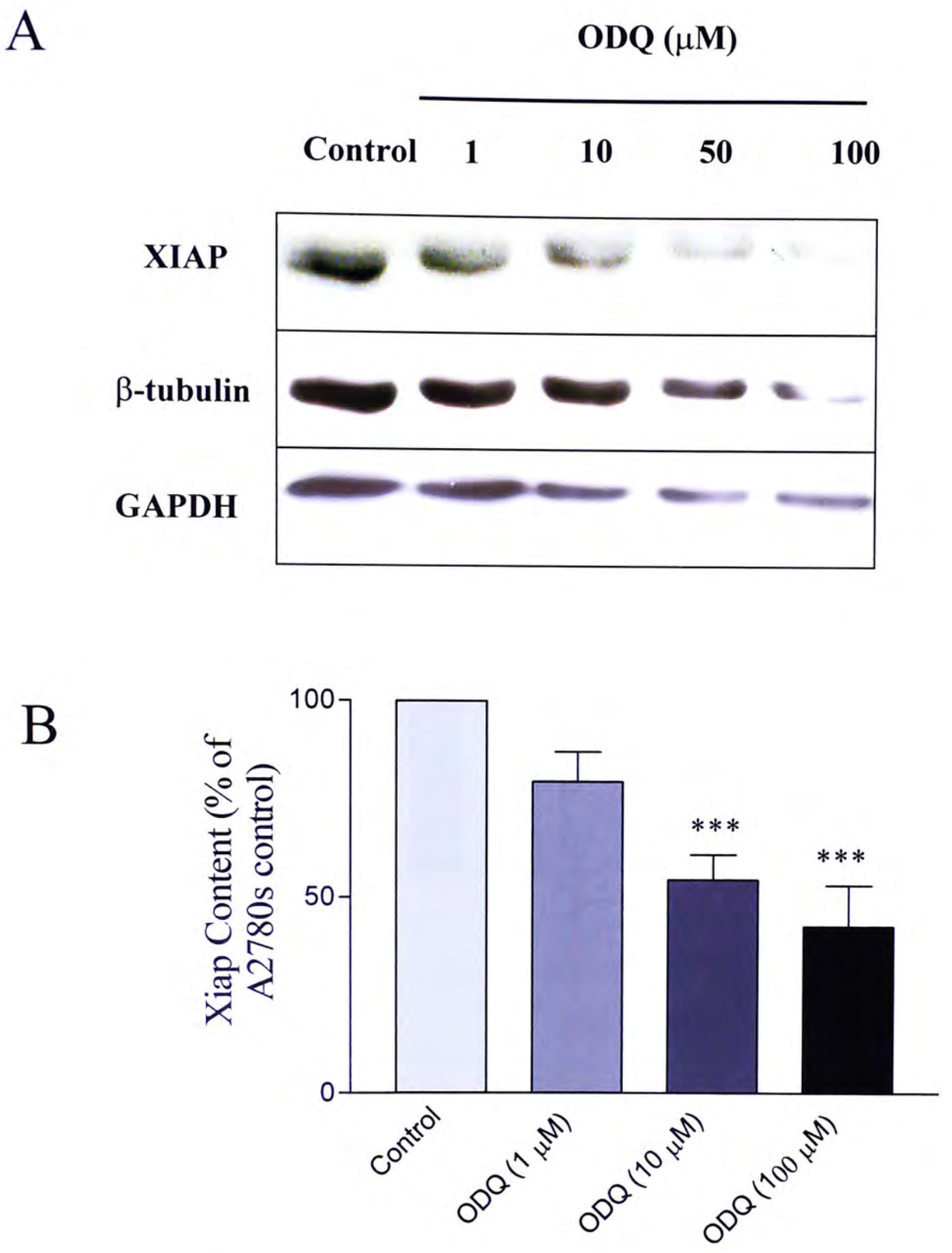


Figure 7.5. Effect of ODQ at different concentrations on XIAP protein content in A2780s cells, assessed by Western blot analysis. (A) Representative blot of the effects of ODQ on XIAP, β-tubulin and GAPDH protein contents. (B) Changes in XIAP protein content, analysed densitometrically using a Molecular Dynamic Phosphoimager. Data represent mean ± SEM of five experients. ***P<0.001 (compared to control).

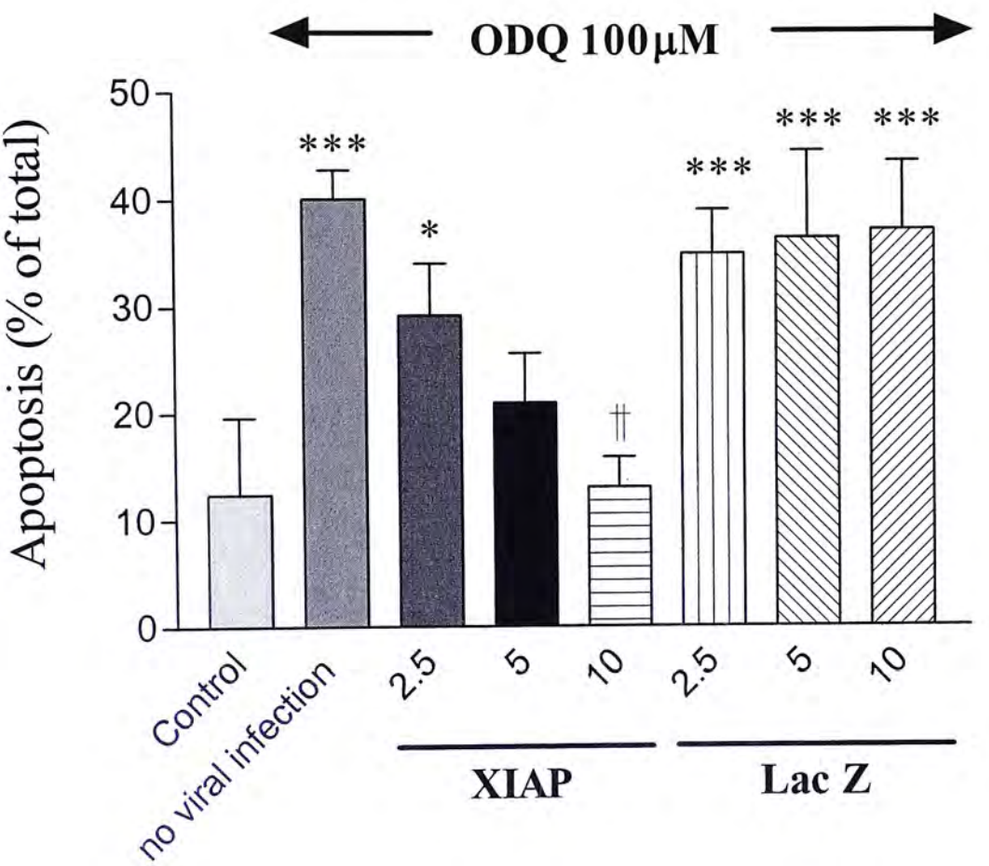
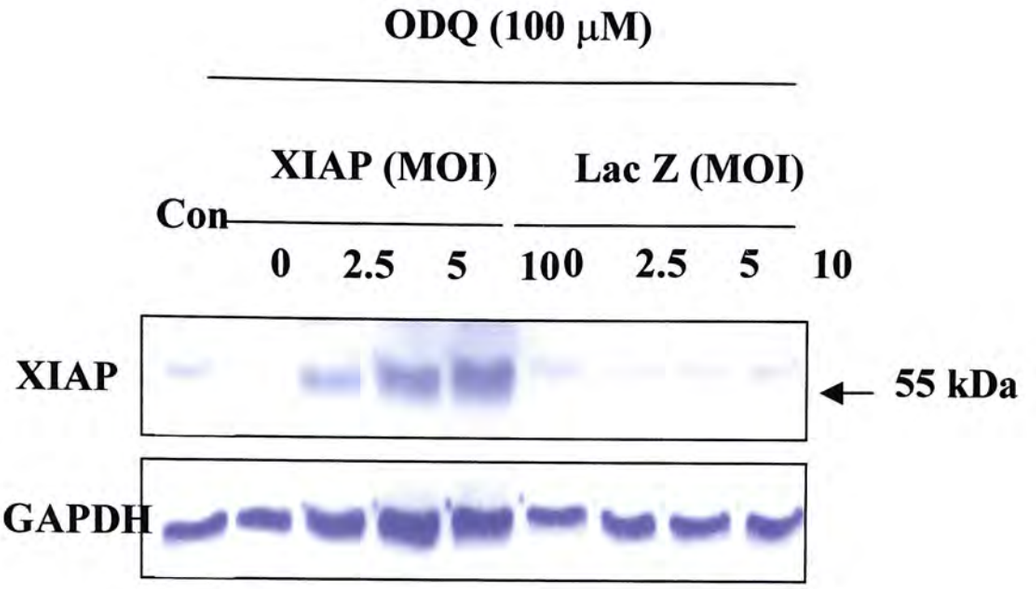


Figure 7.6. Effect of over-expression of XIAP on ODQ-induced apoptosis in A2780s. Cells were plated at a density of 5 x 10^4 cells/cm^2 in 6-well plates for 24 h in DMEM-F12-10% FBS and cultured for 48 h without virus or with adenoviral sense XIAP or Lac Z (MOI=2.5, 5 or 10) vectors, as described in "Materials and Methods". Cells were then treated for 24 h with ODQ (100 μM) and collected for Hoechst staining (for counting the number of apoptotic cells). The graph shows the combined data of three experiments, indicating the dose-dependency of the anti-apoptotic effects of XIAP over-expression on the ODQ-induced apoptosis in the A2780s cells. The data represent the mean ± SEM of n=3 samples per group. *P<0.05 and ***P < 0.001, comparing the treatment groups to the control. ++P<0.01, comparing the treatment group to the ODQ (100 μM)-treated (with no viral infection) group.

A



B

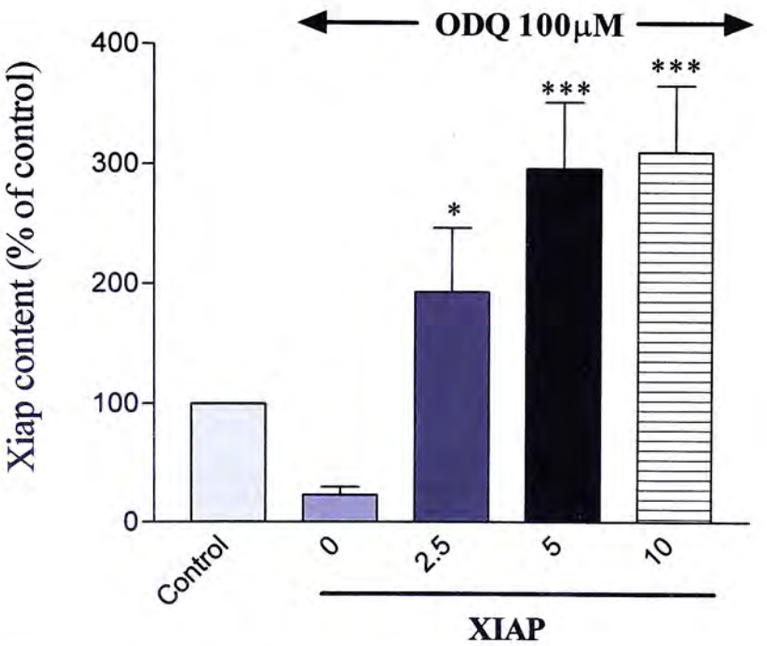


Figure 7.7. Western blot analyses confirming the increases of XIAP protein content in A2780s cells treated with adenoviral sense XIAP or Lac Z infection. (A) Representative blot showing the effect of over-expressing XIAP on the ODQ-induced decreases in XIAP protein contents. (B) Graph indicating the changes in XIAP protein content, analysed densitometrically using a Molecular Dynamic Phosphoimager. The data represent mean ± SEM of three experiments. *P<0.05 and ***P<0.001 (compared to cells treated with 100 μM ODQ but no viral infection).

Chapter 8: Overall conclusions

The present study has shown that exogenous NO, provided by the NO donor SNP, serum deprivation and overcrowded culturing conditions all cause increases in apoptotic DNA fragmentation in uterine epithelial cells, measured by the new quantitative ultrasensitive CE-LIF technique. Using this new technique allowed not only accurate quantification of the apoptotic DNA fragmentation in rabbit immortalized uterine epithelial cells, HRE-H9 cells, but also allowed for the first time quantification of apoptotic DNA fragmentation in a limited number of cells in the primary cultures of mouse uterine epithelial cells.

This new ultrasensitive CE-LIF technique was then used to determine the role of the sGC/cGMP/PKG signaling pathway in the regulation of apoptosis in uterine epithelial cells. The sGC inhibitors NS2028 and ODQ were used to determine the involvement of basal sGC activity and basal levels of cGMP in the regulation of apoptosis. The PKG inhibitor KT5823 was used to determine the involvement of basal PKG activity in the regulation of apoptosis. A non-NO-dependent activator of sGC, YC-1, and direct PKG activators, 8-Br-cGMP, 8-pCPT-cGMP and Sp-8-Br-PET-cGMPS, were used to determine the effects on apoptosis of activating the sGC/cGMP/PKG signaling pathway above basal levels.

The present study has shown that NS2028, ODQ, and KT5823, all cause induction of apoptotic DNA fragmentation in the uterine epitheial cells, HRE-H9 cells. The data suggested that basal levels of sGC and PKG activity are essential to prevent spontaneous development of apoptosis in HRE-H9 cells. Since the addition of the cGMP analog 8-Br-cGMP can prevent the apoptosis induced by ODQ in these cells, the cGMP/PKG siganlling pathways appears to play an important role in the regulation of apoptosis in these immortalized uterine epithelial cells.

After showing that basal levels of cGMP and basal activity of PKG protected uterine epithelial cells against spontaneous development of apoptosis, further experiments were conducted to determine whether activation of the cGMP/PKG pathway above the basal levels could further protect uterine epithelial cells against the development of apoptosis. Addition of direct activators of sGC or PKG, which would activate the cGMP/PKG pathway above the basal levels, did not protect the HRE-H9 cells against apoptosis induced by serum deprivation. Instead, addition of the sGC activator YC-1 or the PKG activators 8-pCPT-cGMP and Sp-8-Bromo-PET-cGMPS significantly enhanced the apoptotic DNA fragmentation induced by serum deprivation. Thus, unlike the protective effects of basal cGMP/PKG activity, the activation of the cGMP/PKG pathway above basal levels appears to promote, rather than inhibit, apoptosis in these immortalized uterine epithelial cells.

The activities of sGC and PKG and the levels of cGMP in uterine epithelial cells are likely to be strictly regulated and to participate in both anti-apoptotic and pro-apoptotic pathways regulating cell survival. Although basal activities of sGC and PKG have anti-apoptotic effects, the elevation of the sGC and PKG activities above basal levels appears to switch the anti-apoptotic effect to a pro-apoptotic effect. Because of the importance of uterine epithelial apoptosis in normal menstruation and fertility and in the pathogenesis of endometriosis, the effect of the cGMP/PKG pathway in the regulation of apoptosis described in the present study may have special importance in the regulation of a number of female reproductive functions.

In addition to uterine epithelial cells, human ovarian cancer cells were also studied as a cell culture model to determine the role of basal activity of sGC in the regulation of apoptosis. The effects of the sGC/cGMP-signaling pathway in the regulation of XIAP expression, which has been demonstrated to suppress apoptosis in these cells, were also determined. The resulting data have shown that the sGC inhibitor ODQ down-regulates XIAP content and induces apoptosis in the human ovarian cancer cell line A2780s. Preventing the decrease in XIAP content by over-expressing this protein completely protected the cells against ODQ-induced apoptosis. Our finding suggest that basal sGC activity and cGMP levels may be necessary for the maintenance of high XIAP level for prevention of spontaneous apoptosis in these ovarian cancer cells under normal culture conditions. Also, these findings provide a new concept for the regulation of apoptosis in

ovarian cancer cells and of ovarian tumor growth, showing a potential link between the anti-apoptic effects of the sGC/cGMP signaling pathway and the cell survival role of XIAP.

Chapter 9: References

Adams J.M., Cory S. The Bcl-2 protein family: arbiters of cell survival. Science. 1998 Aug 28; 281(5381): 1322-1326. Review.

Adayev T., Estephan R., Meserole S., Mazza B., Yurkow E.J., Banerjee P. Externalization of phosphatidylserine may not be an early signal of apoptosis in neuronal cells, but only the phosphatidylserine-displaying apoptotic cells are phagocytosed by microglia. J Neurochem. 1998 Nov; 71(5): 1854-1864.

Albina, J.E., Cui, S., Bateo, R.B., Reichner, J.S. Nitric oxide-mediated apoptosis in murine peritoneal macrophages. J. Immunol. 1993; 150, 5080-5085.

Alnemri E.S., Livingston D.J., Nicholson D.W., Salvesen G., Thornberry N.A., Wong W.W., Yuan J. Human ICE/CED-3 protease nomenclature. Cell. 1996 Oct 18; 87(2): 171.

Ambrosini G., Adida C., Altieri D.C. A novel anti-apoptosis gene, surviving, expressed in cancer and lymphoma. Nat Med. 1997 Aug; 3 (8): 917-921.

Ameisen, J.C. Programmed cell death and AIDS: from hypothesis to experiment. Immunol. Today. 1992; 13(10), 388-391. Review.

Ankarcrona M., Dypbukt J.M., Brune B., Nicotera P. Interleukin-1 beta-induced nitric oxide production activates apoptosis in pancreatic RINm5F cells. Exp Cell Res. 1994 Jul; 213(1): 172-177.

Antonsson B., Martinou J.C. The Bcl-2 protein family. Exp Cell Res. 2000 Apr 10; 256(1): 50-57. Review.

Arstall, M.A., Sawyer, D.B., Fukazawa, R., Kelly, R.A. Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. Circ. Res. 1999; 85, 829-840.

Asselin, E., Mills, G.B. and B.K. Tsang. XIAP regulates Akt activity and caspases-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. Cancer Res. 2001; 61, 1862-1868.

Baker S.J., Reddy E.P. Transducers of life and death: TNF receptor superfamily and associated proteins. Oncogene. 1996 Jan 4; 12(1): 1-9. Review.

Balakirev M. Y., Khramtsov V.V., Zimmer G. Modulation of the mitochondrial permeability transition by nitric oxide. Eur J Biochem. 1997 Jun 15; 246(3): 710-718.

Beauvais, F., Michel, L., Dubertret, L. The nitric oxide donors, azide and hydroxylamine, inhibit the programmed cell death of cytokine-deprived human eosinophils. FEBS Lett. 1995; 361(2-3), 229-232.

Beckman J.S. The double-edged role of nitric oxide in brain function and superoxide-mediated injury. J Dev Physiol. 1991 Jan; 15(1): 53-59.

Beckman J.S., Carson M., Smith C.D., Koppenol W.H. ALS, SOD and peroxynitrite. Nature. 1993 Aug 12; 364(6438): 584.

Beckman, J.S., Koppenol, W.H. Nitric oxide, superoxide and peroxynitrite: the good, the ad, and the ugly. Am. J. Physiol. 271, 1996; C1424-C1437.

Birnbaum M.J., Chlem R.J., Miller L.K. An apoptosis inhibiting gene from a nuclear polyhedrosis virus encoding a peptide with Cys/His sequence motifs. J Virol 1994; 68: 2521-2528.

Blanco, F.J., Ochs, R.L., Schwarz, H., Lotz, M. Chondrocyte apoptosis induced by nitric oxide. Am. J. Pathol. 1995; 146, 75-85.

Bodmer J.L., Burns K., Schneider P., Hofmann K., Steiner V., Thome M., Bornand T., Hahne M., Schroter M., Becker K., Wilson A., French L.E., Browning J.L., MacDonald H.R., Tschopp J. TRAMP, a novel apoptosis-mediating receptor with sequence homology to tumor necrosis factor receptor 1 and Fas (Apo-1/CD95). Immunity. 1997 Jan; 6(1): 79-88.

Bonfoco E., Krainc D., Ankarcrona M., Nicotera P., Lipton S.A. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A. 1995 Aug 1; 92(16): 7162-7166.

Brown D.G., Sun X.M., Cohen G.M. Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. J Biol Chem. 1993 Feb 15; 268(5): 3037-3039.

Brown G.C. Nitric oxide inhibition of cytochrome oxidase and mitochondrial respiration: implications for inflammatory, neurodegenerative and ischaemic pathologies. Mol Cell Biochem. 1997 Sep; 174(1-2): 189-192. Review.

Budihardjo I., Oliver H., Lutter M., Luo X., Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol. 1999; 15:269-290. Review.

Buhimschi, I., Ali, M., Jain, V., Chwalisz, K., Garfield, R.E. Differential regulation of nitric oxide in the rat uterus and cervix during pregnancy and labour. Hum. Reprod. 1996; 11, 1755-1766.

Butt E., Nolte C., Schulz S., Beltman J., Beavo J.A., Jastorff B., Walter U. Analysis of the functional role of cGMP-dependent protein kinase in intact human platelets using a specific activator 8-para-chlorophenylthio-cGMP. Biochem Pharmacol 1992; 43(12): 2591-2600.

Butt E., Geiger J., Jarchau T., Lohmann S.M., Walter U. The cGMP-dependent protein kinase--gene, protein, and function. Neurochem Res. 1993 Jan; 18(1): 27-42. Review.

Butt E., Pohler D., Genieser H.G., Huggins J.P., Bucher B. Inhibition of cyclic GMP-dependent protein kinase-mediated effects by (Rp)-8-bromo-PET-cyclic GMPS. Br J Pharmacol 1995; 116(8): 3110-3116.

Cai J., Yang J., Jones D.P. Mitochondrial control of apoptosis: the role of cytochrome c. Biochim Biophys Acta. 1998 Aug 10; 1366(1-2): 139-149. Review

Castro, A., Johnson, M.C., Anido, M., Cortinez, A., Gabler, F., Vega, M. Role of nitric oxide and bcl-2 family genes in the regulation of human endometrial apoptosis. Fertil Steril. 2002; 78, 587-595.

Ceneviva, G.D., Tzeng, E., Hoyt, D.G., Yee, E., Gallagher, A., Engelhardt, J.F., Kim, Y.M., Billiar, T.R., Watkins, S.A., Pitt, B.R. Nitric oxide inhibits lipopolysaccharide-induced apoptosis in pulmonary artery endothelial cells. Am. J. Physiol. 1998; 275(4 Pt 1), L717-L728.

Chan S.L., Fiscus R.R. ODQ, guanylyl cyclase inhibitor, and KT5823, protein kinase G (PKG) inhibitor, trigger onset of apoptotic DNA fragmentation in uterine epithelial cells: Role of basal cGMP as anti-apoptotic mechanism. In: Program of the 14th World Congress of Pharmacology "The New Century of Pharmacology"; 2002; San Francisco, California, USA. Abstract LB103.

Chan, H.C., Liu, C.Q., Fong, S.K., Law, S.H., Wu, L.J., So, E., Chung, Y.W., Ko, W.H., Wong, P.Y. Regulation of Cl- secretion by extracellular ATP in cultured mouse endometrial epithelium. J Membr Biol. 1997; 156, 45-52.

Chen C.L., Simmen R.C., Chou J.Y. Characterization of endocrine cell lines immortalized by a temperature-sensitive mutant SV40. Chin J Physiol 1991; 34(1): 65-80.

Chen, R.M., Liu, H.C., Lin, Y.L., Jean, W.C., Chen, J.S., Wang, J.H. Nitric oxide induces osteoblast apoptosis through the de novo synthesis of Bax protein. J. Orthop. Res. 2002; 20, 295-302

Cheng W., Li B., Kajstura J., Li P., Wolin M.S., Sonnenblick E.H., Hintze T.H., Olivetti G., Anversa P. Stretch-induced programmed myocyte cell death. J Clin Invest. 1995 Nov; 96(5): 2247-2259.

Cheuk B.L., Chew S.B., Fiscus R.R., Wong P.Y. Cyclooxygenase-2 regulates apoptosis in rat epididymis through prostaglandin D2. Biol Reprod 2002; 66(2): 374-380.

Chiche J.D., Schlutsmeyer S.M., Bloch D.B., de la Monte S.M., Roberts J.D. Jr., Filippov G., Janssens S.P., Rosenzweig A., Bloch K.D. Adenovirus-mediated gene transfer of cGMP-dependent protein kinase increases the sensitivity of cultured vascular smooth muscle cells to the antiproliferative and pro-apoptotic effects of nitric oxide/cGMP. J Biol Chem. 1998 Dec 18; 273(51): 34263-34271.

Chinnaiyan A.M., O'Rourke K., Yu G.L., Lyons R.H., Garg M., Duan D.R., Xing L., Gentz R., Ni J., Dixit V.M. Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95.Science. 1996 Nov 8; 274(5289): 990-992.

Chun, S.Y., Eisenhauer, K.M., Kubo, M. and Hsueh, A.J. Interleukin-1 beta suppresses apoptosis in rat ovarian follicles by increasing nitric oxide production. Endocrinology.1995; 136 (7), 3120-3127.

Cleveland J.L., Ihle J.N. Contenders in FasL/TNF death signaling. Cell. 1995 May 19; 81(4):479-82. Review.

Cohen, G.M. Caspases: The executioners of apoptosis. Biochem J. 1997; 326: 1-16.

Compton M.M. A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. Cancer Metastasis Rev. 1992 Sep; 11(2): 105-119. Review.

Crook N.E., Clem R.J., Miller L.K. An apoptosis-inhibiting baculovirus gene with a zinc fiber-like motif. J Virol 1993; 67: 2168-2174.

Cryns V., Yuan J. Proteases to die for. Genes Dev. 1998 Jun 1; 12(11): 1551-1570. Review.

Dawson V.L., Dawson T.M., Bartley D.A., Uhl G.R., Snyder S.H. Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. J Neurosci. 1993 Jun; 13(6): 2651-2561.

De Caterina R., Libby P., Peng H.B., Thannickal V.J., Rajavashisth T.B., Gimbrone M.A. Jr, Shin W.S., Liao J.K. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. J Clin Invest. 1995 Jul; 96(1): 60-68.

Desagher S., Martinou J.C. Mitochondria as the central control point of apoptosis. Trends Cell Biol. 2000 Sep; 10(9): 369-377. Review

Deveraux Q.L., Takahashi R., Salvesen G.S., Reed J.C. X-linked IAP is a direct inhibitor of cell-death proteases. Nature. 1997 Jul 17; 388 (3339): 300-304

Deveraux Q.L., Roy N., Stennicke H.R., Van Arsdale T., Zhou Q., Srinivasula S.M., Alnemri E.S., Salvesen G.S., Reed J.C. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J. 1998 Apr 15; 17 (8): 2215-2223.

Deveraux Q.L., Leo E., Stennicke H.R., Welsh K., Salvesen G.S., Reed J.C. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J. 1999 Oct 1; 18 (19): 5242-5251.

Dimmeler, S., Haendeler, J., Nehls, M., Zeiher, A.M. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. J. Exp. Med. 1997; 185(4), 601-607.

Duckett C.S., Nava V.E., Gedrich R.W., Clem R.J., Van Dongen J.L., Gilfillan M.C., Shiels H, Hardwick JM, Thompson C.B. A conserved family of cellular genes related to the baculovirus Iap gene and encoding apoptosis inhibitors. EMBO J. 1996 Jun 3; 15 (11): 2685-2694.

Duriez P.J., Shah G.M. Cleavage of poly (ADP-ribose) polymerase: a sensitive parameter to study cell death. Biochem Cell Biol. 1997; 75(4): 337-349. Review.

Duvall E., Wyllie A.H., Morris R.G. Macrophage recognition of cells undergoing programmed cell death (apoptosis). Immunology. 1985 Oct; 56(2): 351-358.

Edwards, Y.S., Sutherland, L.M., Murray, A.W. NO protects alveolar type II cells from stretch-induced apoptosis. A novel role for macrophages in the lung. Am. J. Physiol. Lung Cell. Mol. Physiol. 2000; 279, L1236-L1242.

Estevez, A.G., Radi, R., Barbeito, L., Shin, J.T., Thompson, J.A., Beckman, J.S. Peroxynitrite-induced cytotoxicity in PC12 cells: evidence for an apoptotic mechanism differentially modulated by neurotrophic factors. J. Neurochem. 1995; 65, 1543-1550.

Estevez A.G., Spear N., Thompson J.A., Cornwell T.L., Radi R., Barbeito L., Beckman J.S. Nitric oxide-dependent production of cGMP supports the survival of rat embryonic

motor neurons cultured with brain-derived neurotrophic factor. J Neurosci 1998; 18: 3708-3714.

Estevez, A.G., Spear, N., Manuel, S.M., Barbeito, L., Radi, R. and Beckman, J.S. Role of endogenous nitric oxide and peroxynitrite formation in the survival and death of motor neurons in culture. Prog. Brain Res. 1998; 118, 269-280.

Evans, M.D., Wolfe, J.T., Perrett, D., Lunec, J., Herbert, K.E. Analysis of internucleosomal DNA fragmentation in apoptotic thymocytes by dynamic sieving capillary electrophoresis. J. Chromatogr. A. 1995j; 700, 151-162.

Fernandez-Tome, P., Lizasoain, I., Leza, J.C., Lorenzo, P., Moro, M. A. Neuropretective effects of DETA-NONoate, a nitric oxide donor, on hydrogen peroxide-induced neurotoxicity in cortical neurons. Neuropharmacology. 1999; 38, 1307-1315.

Ferrero R., Torres M. Prolonged exposure to YC-1 induces apoptosis in adrenomedullary endothelial and chromaffin cells through a cGMP-independent mechanism. Neuropharmacology 2001; 41(7): 895-906.

Fiscus, R.R., Rapoport, R.M. and Murad, F. Endothelium-dependent and nitrovasodilator-induced activation of cyclic GMP-dependent protein kinase in rat aorta. J. Cyclic Nucleotide Protein Phosphor. Res. 1983; 9 (6), 415-425.

Fiscus, R.R., Torphy, T.J. and Mayer, S.E. Cyclic GMP-dependent protein kinase activation in canine tracheal smooth muscle by methacholine and sodium nitroprusside. Biochim. Biophys. Acta. 1984; 805(4), 382-392.

Fiscus R.R., Rapoport R.M., Waldman, S.A., Murad F. Atriopeptin II elevates cyclic GMP, activates cyclic GMP-dependent protein kinase, and cause relaxation in rat thoracic aorta. Biochim Biophys Acta 1985; 846: 179-184.

Fiscus R.R. Molecular mechanisms of endothelium-mediated vasodilation. Semin Thromb Hemost 1988; 14 Suppl: 12-22.

Fiscus R.R, Murad F. cGMP-dependent protein kinase activation in intact tissues. Methods Enzymol 1988; 159:150-159.

Fiscus R.R., Ming S.K. Biology of ageing. In: Pang WS, Teoh MK, Ming SK (eds.), Medicine and Surgery in the Older Person. Singapore: Armour Publishing Pte Ltd; 2000: 29-42.

Fiscus R.R., Leung C.P., Yuen J.P., Chan H.C. Quantification of apoptotic DNA fragmentation in a transformed uterine epithelial cell line, HRE-H9, using capillary electrophoresis with laser-induced fluorescence detector (CE-LIF). Cell Biol Internat 2001a; 25 (10): 1007-1011.

Fiscus R.R., Tu A.W., Cheng Chew S.B. Natriuretic peptides inhibit apoptosis and prolong the survival of serum-deprived PC12 cells. Neuroreport 2001b; 12(2): 185-189.

Fiscus, R.R. Involvement of cyclic GMP and protein kinase G in the regulation of apoptosis and survival in neural cells Neurosignals. 2002; 11 (4), 175-190. Review.

Fiscus, R.R., Yuen, J. P., Chan S.L., Kwong, J. H. and Chew, S. B. Nitric oxide and cyclic GMP as pro- and anti-apoptotic agents. J. Card. Surg. 2002; 17 (4), 336-339.

Flamigni F., Facchini A., Stanic I., Tantini B., Bonavita F., Stefanelli C. Control of survival of proliferating L1210 cells by soluble guanylate cyclase and p44/42 mitogen-activated protein kinase modulators. Biochem Pharmacol 2001; 62(3): 319-328.

Friebe A., Schultz G., Koesling D. Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. EMBO J 1996; 15(24): 6863-6868.

Friebe A., Mullershausen F., Smolenski A., Walter U., Schultz G., Koesling D. YC-1 potentiates nitric oxide- and carbon monoxide-induced cyclic GMP effects in human platelets. Mol Pharmacol 1998; 54(6): 962-967.

Fukuo K., Hata S., Suhara T., Nakahashi T., Shinto Y., Tsujimoto Y., Morimoto S., Ogihara T. Nitric oxide induces upregulation of Fas and apoptosis in vascular smooth muscle. Hypertension. 1996 Mar; 27(3 Pt 2): 823-826.

Gabuzda D., Wang J. Chemokine receptors and mechanisms of cell death in HIV neuropathogenesis. J Neurovirol. 2000 May; 6 Suppl 1:S24-S32. Review

Galan, A., Herrer, R., Remohi, J., Pellicer, A. and Simon, C. Embryonic regulation of endometrial epithelial apoptosis during human implantation. Hum. Reprod. 2002; 15 Suppl 6, 74-80.

Galle J., Zabel U., Hubner U., Hatzelmann A., Wagner B., Wanner C., Schmidt H.H. Effects of the soluble guanylyl cyclase activator, YC-1, on vascular tone, cyclic GMP levels and phosphodiesterase activity. Br J Pharmacol. 1999 May; 127(1): 195-203.

Garbers D.L. Guanylyl cyclase receptors and their endocrine, paracrine, and autocrine ligands. Cell 1992; 71 (1): 1-4.

Garcia-Velasco, J.A., Mulayim, N., Kayisli, U.A. and Arici, A. Elevated soluble Fas ligand levels may suggest a role for apoptosis in women with endometriosis. Fertil. Steril. 2002; 78(4), 855-859.

Garthwaite G., Garthwaite J. Cyclic GMP and cell death in rat cerebellar slices. Neuroscience 1988; 26(1): 321-326.

Garthwaite J., Southam E., Boulton C.L., Nielsen E.B., Schmidt K., Mayer B. Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one. Mol Pharmacol. 1995 Aug; 48(2): 184-188.

Gebel, H.M., Braun, D. P., Tambur, A., Frame, D., Rana, N. and Dmowski, W.P. Spontaneous apoptosis of endometrial tissue is impaired in women with endometriosis. Ferile. Steril.1998; 69 (6), 1042-1047.

Genaro, A. M., Hortelano, S., Alvarez A., Martinez, C., Bosca, L. Splenic B lymphocyte programmed cell death is prevented by nitric oxide release through mechanisms involving sustained Bcl-2 levels. J. Clin. Invest. 1995; 95, 1884-1890.

Gompel A., Sabourin J.C., Martin A., Yaneva H., Audouin J., Decroix Y., Poitout P.. Bcl-2 expression in normal endometrium during the menstrual cycle. Am J Pathol. 1994 Jun; 144(6): 1195-1202.

Gosden, R., Spears, N. Programmed cell death in the reproductive system. Br. Bed. Bull. 1997; 53, 644-661. Review.

Gouge, R.C., Marshburn, P., Gordon, B.E., Nunley, W., Huet-Hudson, Y.M. Nitric oxide as a regulator of embryonic development. Biol. Reprod. 1998; 58, 875-879.

Gross A., McDonnell J.M., Korsmeyer S.J. BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 1999 Aug 1; 13(15): 1899-1911. Review.

Gruss H.J., Dower S.K. Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. Blood. 1995 Jun 15; 85(12): 3378-3404. Review.

Hakoda S., Ishikura H., Takeyama N., Tanaka T. Tumor necrosis factor-alpha plus actinomycin D-induced apoptosis of L929 cells is prevented by nitric oxide. Surg Today. 1999; 29(10): 1059-1067.

Hardman, J.G., Limbird, L.E. Goodman & Gilman's The Pharmacological Basis of Therapeutics. 2001; McGraw-Hill Medical Publishing Division, New York, 10th ed. (consulting editor: A.G. Gilman).

Heinloth, A., Brune, B., Fischer, B. Galle, J. Nitric oxide prevents oxidised LDL-induced p53 accumulation, cytochrome c translocation, and apoptosis in macrophages via guanylate cyclase stimulation. Atherosclerosis.2002 162(1), 93-101.

Hortelano S., Dallaporta B., Zamzami N., Hirsch T., Susin S.A., Marzo I., Bosca L., Kroemer G. Nitric oxide induces apoptosis via triggering mitochondrial permeability transition. FEBS Lett. 1997 Jun 30; 410(2-3): 373-377.

Itoh N., Nagata S. A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. J Biol Chem. 1993 May 25; 268(15): 10932-10937.

Jacobson M.D., Weil M., Raff M.C. Programmed cell death in animal development. Cell. 1997 Feb 7; 88(3): 347-354.

Jacobson M.D. Apoptosis: Bcl-2-related proteins get connected. Curr Biol. 1997 May 1; 7(5): R277-R281. Review.

Jaing, Y., Sing, A.K., Kannann, M.S., Johnson, D.E., Shew, R.L. Isolation of nitric oxide synthase in the rat uterus. Life Sci. 1996; 58, 1009-1014.

Janssen Y.M., Matalon S., Mossman B.T. Differential induction of c-fos, c-jun, and apoptosis in lung epithelial cells exposed to ROS or RNS. Am J Physiol. 1997 Oct; 273(4 Pt 1): L789-L796.

Janssen, Y.M., Soultanakis, R., Steece, K., Heerdt, E., Singh, R.J., Joseph, J. and Kalyanaraman, B. Depletion of nitric oxide causes cell cycle alterations, apoptosis, and oxidative stress in pulmonary cells. Am J Physiol. 1998; 275(6 Pt 1), L1100-L1109.

Jedlitschky G., Burchell B., Keppler D. The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. J Biol Chem. 2000 Sep 29; 275(39): 30069-30074.

Kamijo, T., Rajabi, M.R., Mizunuma, H. and Ibuki, Y. Biochemical evidence for autocrine/paracrine regulation of apoptosis in cultured uterine epithelial cells during mouse embryo implantation in vitro. Mol. Hum. Reprod. 1998; 4(10), 990-998.

Kaneto H., Fujii J., Seo H.G., Suzuki K., Matsuoka T., Nakamura M., Tatsumi H., Yamasaki Y., Kamada T., Taniguchi N. Apoptotic cell death triggered by nitric oxide in pancreatic beta-cells. Diabetes. 1995 Jul; 44(7): 733-738.

Kasof G. M., Gomes B.C. Livin, a novel inhibitor of apoptosis protein family member. J Biol Chem. 2001 Feb 2; 276 (5): 3238-3246.

Kelekar A., Thompson C.B. Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol. 1998 Aug;8(8): 324-330. Review.

Kerr J.F., Wyllie A.H., Currie A.R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972 Aug; 26(4): 239-257. Review.

Kerr J. F. R., Winterford C.M., Harmon B.V. Apoptosis: its significance in cancer and cancer therapy. Cancer 1994, 73, 2013-2026.

Kim, Y.M., Talanian, R.V. and Billiar, T.R. Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. J. Biol. Chem. 1997; 272, 31138-31148.

Kim Y.M., Kim T.H., Seol D.W., Talanian R.V., Billiar T.R. Nitric oxide suppression of apoptosis occurs in association with an inhibition of Bcl-2 cleavage and cytochrome c release. J Biol Chem. 1998 Nov 20; 273(47): 31437-31441.

Kim, Y.M., Chung, H.T., Kim, S.S., Han, J.A., Yoo, Y.M., Kim, K.M., Lee, G.H., Yun, H.Y., Green, A., Li, J., Simmons, R.L., Billiar, T.R., 1999. Nitric oxide protects

PC12 cells from serum deprivation-induced apoptosis by cGMP-dependent inhibition of caspase signaling. J. Neurosci. 19, 6740-6747.

Kitson J., Raven T., Jiang Y.P., Goeddel D.V., Giles K.M., Pun K.T., Grinham C.J., Brown R., Farrow S.N. A death-domain-containing receptor that mediates apoptosis. Nature. 1996 Nov 28; 384(6607): 372-375.

Ko F.N., Wu C.C., Kuo S.C., Lee F.Y., Teng C.M. YC-1, a novel activator of platelet guanylate cyclase. Blood 1994; 84(12): 4226-4233.

Kockx M.M., Knaapen M.W.The role of apoptosis in vascular disease. J Pathol. 2000 Feb; 190(3): 267-280. Review.

Kolb, J.P. Mechanisms involved in the pro- and anti-apoptotic role of NO in human leukemia. Leukemia. 2000; 14, 1685-1694.

Kroemer, G., and J.C. Reed. Mitochondrial control of cell death. Nat Med. 2000; 6: 513-519

Kubes P., Suzuki M., Granger D.N. Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci U S A. 1991 Jun 1; 88(11): 4651-4655.

Kure S., Tominaga T., Yoshimoto T., Tada K., Narisawa K. Glutamate triggers internucleosomal DNA cleavage in neuronal cells. Biochem Biophys Res Commun. 1991 Aug 30; 179(1): 39-45.

LaCasse E.C., Baird S., Korneluk R.G., MacKenzie A.E. The inhibitors of apoptosis (IAPS) and their emerging role in cancer. Oncogene 1998, 17, 3247-3259.

Levine E.L., Renehan A., Gossiel R., Davidson S.E., Roberts S.A., Chadwick C., Wilks D.P., Potten C.S., Hendry J.H., Hunter R.D., et al. Apoptosis, intrinsic radiosensitivity and prediction of radiotherapy response in cervical carcinoma. Radiother Oncol 1995, 37, 1-9.

Li, H.Y., Chang, S. P, Yuan, C.C, Chao, H.T., Ng, H. T and Sung, Y.J. Nitric oxide induces extensive apoptosis in endometrial epithelial cells in the presence of progesterone: involvement of mitogen-activated protein kinase pathway. Mol. Hum. Reprod. 2001 7:755-763.

Li J., Billiar T.R. The role of nitric oxide in apoptosis. Semin Perinatol. 2000 Feb; 24(1): 46-50. Review.

Li J., Sasaki H., Sheng Y.L., Schneiderman D., Xiao C.W., Kotsuji F., Tsang B. K. Apoptosis and chemoresistance in human ovarian cancer: is Xiap a determinant? Biol Signals Recept. 2000a Mar-Apr; 9 (2): 122-130. Review.

Li J., Yang S., Billiar T.R.. Cyclic nucleotides suppress tumor necrosis factor alpha-mediated apoptosis by inhibiting caspase activation and cytochrome c release in primary hepatocytes via a mechanism independent of Akt activation. J Biol Chem. 2000 Apr 28; 275(17): 13026-13034.

Li J., Feng Q., Kim J. M., Schneiderman D., Liston P., Li M., Vanderhyden B., Faught W., Fung M.F., Senterman M., Korneluk R. G., Tsang B. K. Human ovarian cancer and cisplatin resistance: possible role of inhibitor of apoptosis proteins. Endocrinology. 2001 Jan; 142 (1): 370-380.

Li W.I., Chen C.L., Chou J.Y. Characterization of a temperature-sensitive beta-endorphin-secreting transformed endometrial cell line. Endocrinology 1989; 125(6): 2862-2867.

Liebermann D.A., Hoffman B., Steinman R.A. Molecular controls of growth arrest and apoptosis: p53-dependent and independent pathways. Oncogene. 1995 Jul 6; 11(1): 199-210. Review.

Lin, K.T., Xue, J.Y., Nomen, M., Spur, B., Wong, P.Y. Peroxynitrite-induced apoptosis in HL-60 cells. J. Biol. Chem. 1995; 270, 16487-16490.

Lincoln T.M., Cornwell T.L. Intracellular cyclic GMP receptor proteins. FASEB J 1993; 7 (2): 328-338.

Lincz L.F. Deciphering the apoptotic pathway: all roads lead to death. Immunol Cell Biol. 1998 Feb; 76(1): 1-19. Review.

Lipton S.A., Choi Y.B., Pan Z.H., Lei S.Z., Chen H.S., Sucher N.J., Loscalzo J., Singel D.J., Stamler J.S. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature. 1993 Aug 12; 364(6438): 626-632.

Liston P., Roy N., Tamai K., Lefebvre C. Baird S., Cherton-Horvat G., Farahani R., McLean M., Ikeda J. E., MacKenzie A., Korneluk R.G. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature. 1996 Jan 25; 379 (6563): 349-353.

Liu L., Li H., Underwood T., Lloyd M., David M., Sperl G., Pamukcu R., Thompson W.J. Cyclic GMP-dependent protein kinase activation and induction by exisulind and CP461 in colon tumor cells. J Pharmacol Exp Ther 2001; 299(2): 583-592.

Loweth A.C., Williams, G.T., Scarpello, J.H., Morgan, G. Evidence for the involvement of cGMP and protein kinase G in nitric oxide-induced apoptosis in the pancreatic B-cell line, HIT-T15. FEBS Lett. 1997; 400, 285-288.

Luke R., Sharpe N., Coxon R. Transdermal nitroglycerin in angina pectoris: efficacy of intermittent application. J Am Coll Cardiol. 1987 Sep; 10(3): 642-646.

Mahidhara R., Billiar T.R. Apoptosis in sepsis. Crit Care Med. 2000 Apr; 28(4 Suppl): N105-N113. Review.

Mannick, J. B., Asano, K., Izumi, K., Kieff, EI. And Stamler, J.S. Nitric oxide produced by human B-lymphocytes inhibits apoptosis and Epstein-Barr virus re-activation. Cell. 1994; 79(7), 1137-1146.

Marsters S.A., Sheridan J.P., Donahue C.J., Pitti RM, Gray C.L., Goddard A.D., Bauer K.D., Ashkenazi A. Apo-3, a new member of the tumor necrosis factor receptor family, contains a death domain and activates apoptosis and NF-kappa B. Curr Biol. 1996 Dec 1; 6(12): 1669-1676.

McCormack, S.A., Glasser, S.R., 1980. Differential response of individual uterine cell types from immature rats treated with estradial. Endocrinology. 106, 1634-1649.

Meinhardt, G., Wendtner, C.M. and Hallek M. Molecular pathogenesis of chronic lymphocytic leukemia: factors and signiling pathways regulating cell growth and survival. J. Mol. Med. 1999; 77, 282-293.

Melino G., Bernassola F., Knight R.A., Corasaniti M.T., Nistico G., Finazzi-Agro A. S-nitrosylation regulates apoptosis. Nature. 1997 Jul 31; 388(6641): 432-433.

Moncada S., Palmer R.M., Higgs E.A. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev. 1991 Jun; 43(2): 109-142. Review.

Moro, M.A., Russel, R.J., Cellek, S., Lizasoain, I., Su, Y., Darley-Usmar, V.M., Radomski, M.W. and Moncada, S. cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase. Proc. Natl. Acad. Sci. U. S. A. 1996; 93(4), 1480-1485.

Mulsch A., Bauersachs J., Schafer A., Stasch J.P., Kast R., Busse R. Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators. Br J Pharmacol 1997; 120(4): 681-689.

Nagata S. Apoptotic DNA fragmentation. Exp Cell Res. 2000 Apr 10; 256(1): 12-18. Review.

Nagata S., Golstein P. The Fas death factor. Science. 1995 Mar 10; 267(5203): 1449-1456. Review

Nakagawa T., Yuan J. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J Cell Biol. 2000 Aug 21; 150(4): 887-894.

Nakagawa T., Zhu H., Morishima N., Li E., Xu J., Yankner B.A., Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature. 2000 Jan 6; 403(6765): 98-103.

Newton K., Strasser A. The Bcl-2 family and cell death regulation. Curr Opin Genet Dev. 1998 Feb;8(1): 68-75. Review

Neyns B., Katesuwanasing., Vermeij J., Bourgain C., Vandamme B., Amfo K., Lissens W., DeSutter P., Hooghe-Peters E., DeGreve J. Expression of the jun family of genes in human ovarian cancer and normal ovarian surface epithelium. Oncogene 1996; 12: 1247-1257.

Nguyen T, Brunson D, Crespi CL, Penman BW, Wishnok JS, Tannenbaum SR. DNA damage and mutation in human cells exposed to nitric oxide in vitro. Proc Natl Acad Sci U S A. 1992 Apr 1; 89(7): 3030-3034.

Nicholson D.W., Thornberry N.A. Caspases: killer proteases. Trends Biochem Sci. 1997 Aug; 22(8): 299-306. Review.

Nishio, E., Fukushima, K., Shiozaki, M., Watanabe, Y. Nitric oxide donor SNAP induces apoptosis in smooth muscle cells through cGMP-independent mechanism. Biochem Biophys Res. Commun. 1996; 221, 163-168.

O'Connor, M., Salzman, A.L., Szabo, C. Role of peroxynitrite in the protein oxidation and apoptotic DNA fragmentation in vascular smooth muscle cells stimulated with bacterial lipopolysaccharide and interferon-gamma. Shock. 1997; 8, 439-443.

Olesen, S.P., Drejer, J., Axelsson, O., Moldt, P., Bang, L., Nielsen-Kudsk, J.E., Busse, R. and Mulsch, A. Characterization of NS 2028 as a specific inhibitor of soluble guanylyl cyclase. Br. J. Pharmacol.1998; 123(2), 299-309.

Otsuki Y., Misaki O., Sugimoto O., Ito Y., Tsujimoto Y., Akao Y. Cyclic bcl-2 gene expression in human uterine endometrium during menstrual cycle. Lancet. 1994 Jul 2; 344(8914): 28-29.

Ou J., Carlos T.M., Watkins S.C., Saavedra J.E., Keefer L.K., Kim Y.M., Harbrecht B.G., Billiar T.R. Differential effects of nonselective nitric oxide synthase (NOS) and selective inducible NOS inhibition on hepatic necrosis, apoptosis, ICAM-1 expression, and neutrophil accumulation during endotoxemia. Nitric Oxide. 1997 Oct; 1(5): 404-416.

Pai, N., Zdanski, C.J., Gregory, C.W., Prazma, J., Carrasco, V. Sodium nitroprusside/nitric oxide causes apoptosis in spiral ganglion cells. Otolaryngol. Head Neck Surg. 1998j; 119, 323-330.

Pampfer S., Donnay I. Apoptosis at the time of embryo implantation in mouse and rat. Cell Death Differ. 1999 Jun; 6(6): 533-545. Review.

Pantazis, N. J., West, J. R., Dai, D. The nitric oxide-cyclic GMP pathway plays an essential role in both promoting cell survival of cerebellar granule cells in culture and protecting the cells against ethanol neurotoxicity. J. Neurochem. 1998; 70, 1826-1838.

Parr, E.L., Tung, H.N. and Parr, M.B. Apoptosis as the mode of uterine epithelial cell death during embryo implantation in mice and rats. Biol. Reprod. 1987; 36(1), 211-225.

Patel M.J., Wypij D.M., Rose D.A., Rimele T.J., Wiseman J.S. Secretion of cyclic GMP by cultured epithelial and fibroblast cell lines in response to nitric oxide. J Pharmacol Exp Ther. 1995 Apr; 273(1): 16-25.

Pollman MJ, Yamada T, Horiuchi M, Gibbons GH. Vasoactive substances regulate vascular smooth muscle cell apoptosis. Countervailing influences of nitric oxide and angiotensin II.Circ Res. 1996 Oct; 79(4): 748-756.

Pospisil R., Young-Cooper G.O., Mage R.G. Preferential expansion and survival of B-lymphocytes based on VH framework 1 and framework 3 expression: "positive" selection in appendix of normal and VH-mutant rabbits. Proc Natl Acad Sci U S A. 1995 Jul 18; 92(15): 6961-6965.

Potter, C.L., Hanson, P.J. Exogenous nitric oxide inhibits apoptosis in guinea pig gastric mucous cells. Gut. 2000; 46, 156-162.

Rabinovitch A., Suarez-Pinzon W.L., Shi Y., Morgan A.R., Bleackley R.C. DNA fragmentation is an early event in cytokine-induced islet beta-cell destruction. Diabetologia. 1994 Aug; 37(8): 733-738.

Radomski M.W., Palmer R.M., Moncada S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci U S A. 1990 Jul; 87(13): 5193-5197.

Radomski M.W., Palmer R.M., Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. Lancet. 1987 Nov 7; 2(8567): 1057-1058.

Radomski M.W., Salas E. Nitric oxide--biological mediator, modulator and factor of injury: its role in the pathogenesis of atherosclerosis. Atherosclerosis. 1995 Dec; 118 Suppl: S69-S80. Review.

Raff M.C., Size control: the regulation of cell numbers in animal development. Cell. 1996 Jul 26; 86(2): 173-175. Review

Reap E.A., Leslie D., Abrahams M., Eisenberg R.A., Cohen P.L. Apoptosis abnormalities of splenic lymphocytes in autoimmune lpr and gld mice.J Immunol. 1995 Jan 15; 154(2): 936-943.

Reed J. Bcl-2 family proteins. Oncogene. 1998 Dec 24; 17(25): 3225-3236. Review.

Reed J.C. Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. Semin Hematol. 1997 Oct; 34(4 Suppl 5): 9-19. Review

Rossig L., Fichtlscherer B, Breitschopf K, Haendeler J, Zeiher AM, Mulsch A, Dimmeler S. Nitric oxide inhibits caspase-3 by S-nitrosation in vivo. J Biol Chem. 1999 Mar 12; 274(11): 6823-6826.

Rotello, R.J., Hocker, M.B., Gerschenson, L.E. Biochemical evidence for programmed cell death in rabbit uterine epithelium. Am. J. Pathol. 1989; 134, 491-495.

Rothe M., Pan M. G., Henzel W.J, Ayres T.M., Goeddel D.V. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell. 1995 Dec 29; 83 (7): 1243-1252

Roy N., Mahadevan M.S., McLean M., Shutler G., Yaraghi Z., Farahani R., Baird S., Besner-Johnston A., Lefebvre C., Kang X., Salih M., Aubry H., Tamai K., Guan X., Ioannou P., Crawford To, Jong P.J.D., Korneluk R.G. MacKenzie A: The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. Cell. 1995 Jan 13; 80(1): 167-178.

Roy N., Deveraux Q.L., Takahashi R., Salvesen G.S., Reed J.C. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J. 1997 Dec 1; 16(23): 6914-6925.

Salgo, M.G., Squadrito, G.L., Pryor, W.A. Peroxynitrite causes apoptosis in rat thymocytes. Biochem Biophys Res. Commun.1995; 215, 1111-1118.

Sandau, K., Pfeilschifter, J., Brune, B. Nitric oxide and superoxide induced p53 and Bax accumulation during mesangial cell apoptosis. Kidney Int. 1997; 52, 378-386.

Sarih, M., Souvannavong, V., Adam, A. Nitric oxide synthase induces macrophage death by apoptosis. Biochem. Biophys. Res. Commun. 1993; 191, 503-508.

Sasaki H., Sheng Y., Kotsuji F., Tsang B.K. Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. Cancer Res. 2000 Oct 15; 60(20): 5659-5666.

Sato, T., Fukazawa, Y., Kojima, H., Enari, M., Iguchi, T. and Ohta, Y. Apoptotic cell death during the estrous cycle in the rat uterus and vagina. Anat. Rec. 1997; 248 (1), 76-83.

Schneiderman D., Kim J.M., Senterman M and Tsang, B. K. Sustained suppression of Fas ligand expression in cisplatin-resistant human ovarian surface epithelial cancer cells. Apoptosis, 1999; 4: 271-282.

Searle J., Kerr J.F., Bishop C.J. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol Annu. 1982; 17 Pt 2:229-259.

Sekhar K.R., Hatchett R.J., Shabb J.B., Wolfe L., Francis S.H., Wells J.N., Jastorff B., Butt E., Chakinala M.M., Corbin J.D. Relaxation of pig coronary arteries by new and potent cGMP analogs that selectively activate type I alpha, compared with type I beta, cGMP-dependent protein kinase. Mol Pharmacol. 1992 Jul; 42(1): 103-108.

Sheets E.E., Yeh J. The role of apoptosis in gynaecologic malignancies. Ann med 1997, 29, 121-126.

Shimojo, T., Hiroe, M., Ishiyama, S., Ito, H., Nishikawa, T., Marumo, F. Nitric oxide induces apoptotic death of cardiomyocytes via a cyclic-GMP-dependent pathway. Exp. Cell Res. 1999; 247, 38-47.

Sidoti-de Fraisse C, Rincheval V, Risler Y, Mignotte B, Vayssiere JL. TNF-alpha activates at least two apoptotic signaling cascades. Oncogene. 1998 Oct 1; 17(13): 1639-1651.

Siles, B., Nackerdien Z.E., Collier., G.B. Analysis of DNA fragmentation using a dynamic size-sieving polymer solution in capillary electrophoresis. J. Chromatogr A. 1997; 771, 319-329.

Smith C.A., Farrah T., Goodwin R.G. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell. 1994 Mar 25; 76(6): 959-962. Review.

Soh J.W., Mao Y., Liu L., Thompson W.J., Pamukcu R., Weinstein I.B. Protein kinase G activates the JNK1 pathway via phosphorylation of MEKK1. J Biol Chem 2001; 276(19): 16406-16410.

Solis-Recendez, M.G., Perani, A., D'Habit, B., Stacey, G.N., Maugras, M. Hybridoma cell cultures continuously undergo apoptosis and reveal a novel 100 bp DNA fragment. J. Biotechnol. 1995; 38, 117-127.

Srinivasan A., Roth K.A., Sayers R.O., Shindler K.S., Wong A.M., Fritz L.C., Tomaselli K.J. In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. Cell Death Differ. 1998 Dec; 5(12): 1004-1016.

Steller H. Mechanisms and genes of cellular suicide. Science. 1995 Mar 10; 267(5203): 1445-1449. Review.

Suenobu, N., Shichiri, M., Iwashina, M., Marumo, R., Hirata, Y. Natriuretic peptides and nitric oxide induce endothelial apoptosis via a cGMP-dependent mechanism. Arterioscler Thromb Vasc Biol. 1999; 19, 140-146.

Susin S.A., Zamzami N., Kroemer G. Mitochondria as regulators of apoptosis: doubt no more. Biochim Biophys Acta. 1998 Aug 10; 1366(1-2): 151-165. Review

Tabibzadeh, S. Signals and molecular pathways involved in apoptosis, with special emphasis on human endometrium. Hun. Reprod. Update. 1995; 1(4), 303-323. Review.

Taimor G., Hofstaetter B., Piper H.M. Apoptosis induction by nitric oxide in adult cardiomyocytes via cGMP-signaling and its impairment after simulated ischemia. Cardiovasc Res. 2000 Feb; 45(3): 588-594.

Takahashi R., Deveraux Q. Tamm I., Welsh K., Assa-Munt N., Salvesen G.S., Reed J.C. A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem. 1998 Apr 3; 273 (14): 7787-7790.

Takuma, K., Phuagphong, P., Lee, E., Mori, K., Baba, A. and Matsuda, T. Antiapoptotic effect of cGMP in cultured astrocytes: Inhibition by cGMP-dependent protein kinase of mitochondrial permeable transition pore. J. Biol. Chem. 2001; 276 (51), 48093-48099.

Tao, X.J., Tilly, K.I., Maravei, D.V., Shifren, J.L., Krajewski, S., Reed, J.C., Tilly, J.L., Isaacson, K.B. Differential expression of members of the bcl-2 gene family in proliferative and secretory human endometrium: glandular epithelial cell apoptosis is associated with increased expression of bax. J. Clin. Endocrinol. Metab. 1997; 82, 2738-2746.

Tartaglia L.A., Ayres T.M., Wong G.H., Goeddel D.V. A novel domain within the 55 kd TNF receptor signals cell death. Cell. 1993 Sep 10; 74(5): 845-853.

Tejedo, J.R., Ramirez, R., Cahuana, G.M., Rincon, P., Sobrino, F. and Bedoya, F.J. Evidence for involvement of c-Src in the anti-apoptotic action of nitric oxide in serum-deprived RINm5F cells. Cell Signal.2001; 13(11), 809-817.

Telfer, J.F., Irvine, G.A., Kohnen, G., Campbell, S., Cameron, I.T. Expression of endothelial and inducible nitric oxide synthase in non-pregnant and decidualized human endometrium. Mol. Hum. Reprod. 1997; 3, 69-75.

Tenneti L, D'Emilia DM, Lipton SA.Suppression of neuronal apoptosis by S-nitrosylation of caspases. Neurosci Lett. 1997 Nov 7; 236(3): 139-142.

Thadani U. Secondary preventive potential of nitrates in ischaemic heart disease. Eur Heart J. 1996 Dec; 17 Suppl F: 30-36. Review.

Thippeswamy T., Morris R. Cyclic guanosine 3', 5' -monophosphate-mediated neuroprotection by nitric oxide in dissociated cultures of rat dorsal root ganglion neurons. Brain Res 1997; 774: 116-122.

Thippeswamy, T., Mckay, J. S., Morris, R. Bax and caspases are inhibited by endogenous nitric oxide in dorsal root ganglion neurons in vitro. Eur. J. Neurosci. 2001; 14, 1229-1236.

Thompson C.B. Apoptosis in the pathogenesis and treatment of disease. Science. 1995 Mar 10; 267(5203): 1456-1462. Review.

Thompson W.J., Piazza G.A., Li H., Liu L., Fetter J., Zhu B., Sperl G., Ahnen D., Pamukcu R. Exisulind induction of apoptosis involves guanosine 3', 5'-cyclic

monophosphate phosphodiesterase inhibition, protein kinase G activation, and attenuated beta-catenin. Cancer Res 2000; 60(13): 3338-3342.

Toki, T., Mori, A., Shimizu, M., Nikaido, T. and Fujii, S. Localization of apoptotic cells within the human endometrium and correlation between apoptosis and p21 expression. Mol. Hum. Reprod. 1998; 4(12), 1157-1164.

Troy C.M., Derossi D., Prochiantz A., Greene L.A., Shelanski M.L. Downregulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide-peroxynitrite pathway. J Neurosci. 1996 Jan; 16(1): 253-261.

Tschugguel, W., Schneeberger, C., Unfried, G., Brautigam, G., Stonek, F., Wieser, F., Vytiska-Binstorfer, E., Czerwenka, K., Weninger, W., Kaider, A., Bursch, W., Breitschopf, H., Huber, J.C. Elevation of inducible nitric oxide synthase activity in human endometrium during menstruation. Biol Reprod.1999; 60, 297-304.

Tsujimoto Y., Cossman J., Jaffe E., Croce C.M. Involvement of the bcl-2 gene in human follicular lymphoma. Science. 1985 Jun 21; 228(4706): 1440-1443.

Uren A.G., Pakusch M., Hawkins C.J., Puls K.L., Vaux D.L. Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. Proc Natl Acad Sci U S A. 1996 May 14;93(10):4974-4978.

Vaandrager A.B., Smolenski A., Tilly B.C., Houtsmuller A.B., Ehlert E.M., Bot A.G., Edixhoven M., Boomaars W.E., Lohmann S.M., de Jonge H.R. Membrane targeting of cGMP-dependent protein kinase is required for cystic fibrosis

transmembrane conductance regulator Cl- channel activation. Proc Natl Acad Sci USA 1998; 95(4): 1466-1471.

Waldman, S.A., and Murad, F. (1987) Cyclic GMP synthesis and function. *Pharmacol Rev.* 39, 163-196.

Wakahara Y., Nawa A., Okamoto T., Hayakawa A., Kikkawa F., Suganama N., Wakahara F., Tomoda Y. Combination effect of anti-Fas antibody and chemotherapeutic drugs in ovarian cancer cells in vitro. Oncology. 1997 Jan-Feb;54(1):48-54.

Wei J.Y., Cohen E.D., Yan Y.Y., Genieser H.G., Barnstable C.J. Identification of competitive antagonists of the rod photoreceptor cGMP-gated cation channel: beta-phenyl-1, N2-etheno-substituted cGMP analogues as probes of the cGMP-binding site. Biochemistry 1996; 35(51): 16815-16823.

Wei J.Y., Cohen E.D., Genieser H.G., Barnstable C.J. Substituted cGMP analogs can act as selective agonists of the rod photoreceptor cGMP-gated cation channel. J Mol Neurosci 1998; 10 (1): 53-64.

Wing, L.Y., Hsiet, W.J. and Yang, M.R. Ovarian steroids reduced apoptotic death in SV40 temperature-sensitive mutant virus transformed uterine epithelial cells. Life Sci. 1998; 62(11), 981-987.

Wingo, P.A., Tong, T., Bolden, S. Cancer statistics. CA Cancer J. Clin. 1998; 45: 8-30.

Wohlfart P., Malinski T., Ruetten H., Schindler U., Linz W., Schoenafinger K., Strobel H., Wiemer G. Release of nitric oxide from endothelial cells stimulated by YC-1, an activator of soluble guanylyl cyclase. Br J Pharmacol 1999; 128(6): 1316-1322

Wong A.S., Kim S.O., Leung P.C., Auersperg N., Pelech S.L. Profiling of protein kinases in the neoplastic transformation of human ovarian surface epithelium. Gynecol Oncol 2001; 82(2): 305-311.

Wong B., Choi Y. Pathways leading to cell death in T cells. Curr Opin Immunol. 1997 Jun;9(3):358-364. Review

Wu C.C., Ko F.N., Kuo S.C., Lee F.Y., Teng C.M. YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase. Br J Pharmacol 1995; 116(3): 1973-1978.

Wu C.F., Bishopric N.H., Pratt R.E. Atrial natriuretic peptide induces apoptosis in neonatal rat cardiac myocytes. J Biol Chem. 1997 Jun 6; 272(23): 14860-14866.

Wyatt T.A., Spurzem J.R., May K., Sisson J.H. Regulation of ciliary beat frequency by both PKA and PKG in bovine airway epithelial cells. Am J Physiol 1998; 275(4 Pt 1): L827-L835.

Wyllie A.H., Kerr J.F., Currie A.R. Cell death: the significance of apoptosis. Int Rev Cytol. 1980; 68:251-306. Review.

Wyllie A.H. Apoptosis: cell death in tissue regulation. J Pathol. 1987 Dec; 153(4): 313-316. Review.

Yamada, M., Momose, K., Richelson, E., Yamada, M. Sodium nitroprusside-induced apoptotic cellular death via production of hydrogen peroxide in murine neuroblastoma N1E-115 cells. J. Pharmacol. Toxicol. Methods. 1996; 35, 11-17.

Yang Y, S., Fang, J. P., Jensen, A. M., Weissman, J. D. Ashwell: Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science 2000; 288, 874-877

Yuan J. Transducing signals of life and death. Curr Opin Cell Biol. 1997 Apr; 9(2): 247-251. Review.

Yuen, J.P., Fiscus, R.R. ODQ, a soluble guanylyl cyclase inhibitor, increases the neurotoxicity of nitric oxide (NO) donor and, by itself, causes the onset of apoptotic DNA fragmentation in the NG108-15 neuronal cell line. Cell Biol. Internat. 2001; 25(10): 1073.

Zamora R., Alarcon L., Vodovotz Y., Betten B., Kim P.K., Gibson K.F., Billiar T.R. Nitric oxide suppresses the expression of Bcl-2 binding protein BNIP3 in hepatocytes. J Biol Chem. 2001 Dec 14; 276(50): 46887-16895.